Doctoral Dissertation

Inactivation of planktonic and biofilm cells by copper-based hybrid disinfection systems: Application to biofouling control on RO membranes

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(Environmental Science and Engineering)

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ABSTRACT

Development of biofilm is easily found in any moist environment, and it causes a number of problems, especially financial losses in the industrial field, when it occurs in undesired places, such as membranes, heat exchanger systems, ship hulls, and drinking water distribution systems. In particular, the formation of biofilm on the membrane surface (biofouling) is one of the biggest challenges to the performance of the reverse osmosis (RO) process. In this dissertation, three different types of copper-based disinfection systems including oxidative disinfectants, biocides, and signal molecule were investigated to control biofilm formed on RO membrane.

The first part of the dissertation investigates combinations of Cu(II) with hydroxylamine (HA) and hydrogen peroxide (H_2O_2) (i.e., Cu(II)/HA, Cu(II)/H_2O_2, and Cu(II)/HA/H_2O_2 systems) for the control of *Pseudomonas aeruginosa* biofilms on RO membranes. These Cu(II)-based disinfection systems effectively inactivated *P. aeruginosa* cells, exhibiting different behaviors depending on the state of bacterial cells (planktonic or biofilm) and the condition of biofilm growth and treatment (normal or pressurized condition). The Cu(II)/HA and Cu(II)/HA/H_2O_2 systems were the most effective reagents for the inactivation of planktonic cells. However, these systems were not effective in inactivating cells in biofilms on the RO membranes possibly due to the interactions of Cu(I) with extracellular polymeric substances (EPS), where biofilms were grown and treated in center for disease control (CDC) reactors. Differ from the results using CDC reactors, in a pressurized cross-flow RO filtration unit, the Cu(II)/HA/H_2O_2 treatment significantly inactivated biofilm cells formed on the RO membranes, successfully recovering the permeate flux reduced by the biofouling. The pretreatment of feed solutions by Cu(II)/HA and Cu(II)/HA/H_2O_2 systems (applied before the biofilm formation) effectively mitigated the permeate flux decline by preventing the biofilm growth on the RO membranes.

The second part of the dissertation utilizes the copper-based system (Cu(II), Cu(II)/HA), which is known as an effective biocide, with norspermidine (Nspd) as a disassembly reagent to control biofilm formed on membrane, and evaluates its potential as a cleaning reagent for biofouling control. Combination with Nspd results in improved inactivation efficacy of the copper-based system in biofilm. In particular, the Cu(II)/HA/Nspd (2.0 log in 10 min) system showed significant enhancement compared to the Cu(II)/HA system (4.2 log in 5 min). The results indicated that disruption of EPS by Nspd contributed to higher penetration of copper rather than detachment of cells under the applied conditions. Furthermore, our finding showed that the combined system is applicable in practical conditions (under pressurized condition) with capable biofilm inactivation efficacy, and a valid degree of permeate flux recovery was also observed, approving the feasibility as a cleaning reagent for biofouling control.

The third part of the dissertation assesses the biocidal effects of the Cu(II)-activated persulfate in

the presence of chloride ion (i.e., PMS, Cu(II), Cu(II)/PMS, PMS/Cl^-, and Cu(II)/PMS/Cl^- systems) on planktonic and biofilm cells, and evaluates the feasibility of these disinfection systems in the RO desalination process. The $SO_4^{\cdot -}$ was found to be the main produced oxidant via the Cu(II)-activated persulfate system. In addition, the production of sulfate radial likely occurred close to bacteria cells where the Cu(I) was interred, and the produced bacteria-bound sulfate radical would directly damage *P. aeruginosa* cells. The enhanced inactivation efficacy in the Cu(II)/PMS/Cl^- system was attributed to the production of reactive species by a dual mechanism through the Cu(II)/PMS system and the Cu(II)/in situ production of HOCl system.

Contents

List of figures

List of tables

I. INTRODUCTION

1.1 Problem statement

The matrix of extracellular polymeric substances (EPS) enclosing cells presents a difficult hurdle in the inactivation of biofilms. It has been reported that cells in biofilms are up to 1000-fold more resistant to disinfectants than planktonic cells (Kim et al., 2008; Masak et al., 2014). Biofilms are ubiquitous and problematic in many industrial fields, including water distribution systems, heat exchangers, ship hulls, and membrane filters (Flemming, 2002). In particular, the formation of biofilms on reverse osmosis (RO) membranes (biofouling) in desalination plants significantly lowers the performance of these membranes and is recognized as the largest obstacle in the RO process, although RO process is admitted as the most cost-effective technology for desalination (Mansouri et al., 2010; Matin et al., 2011).

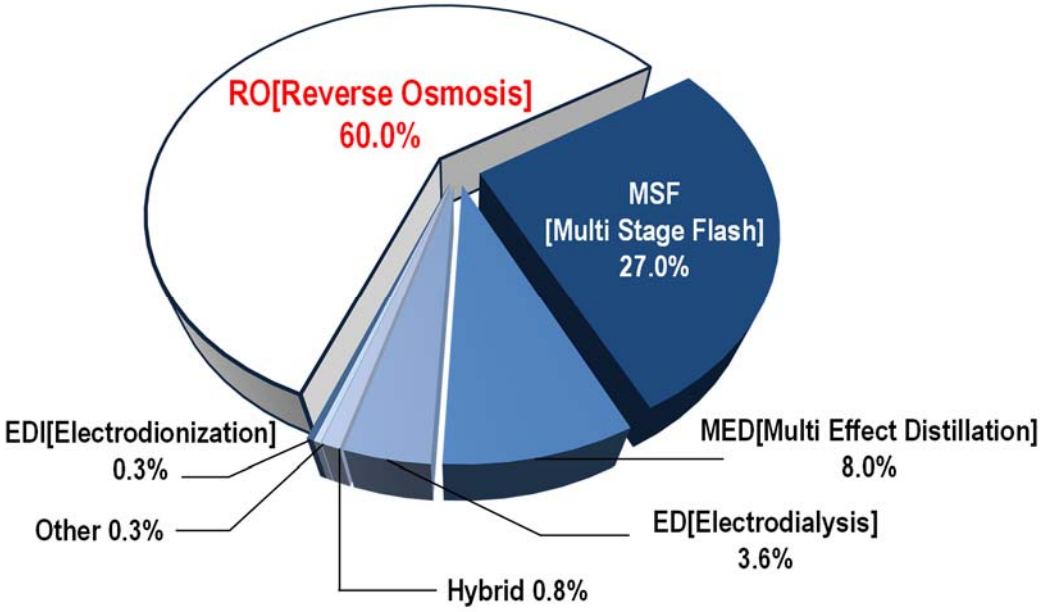

Figure 1. 1 *Desalination technology market*

To alleviate the biofouling of the RO membranes, chlorine is frequently added in the pretreatment step and then quenched immediately preceding the RO process (Kwon and Leckie, 2006). Unfortunately, improper quenching of chlorine can cause serious damage to polyamide active layers of RO membranes. Furthermore, the potential health risks associated with transport and storage of chlorine, as well as the formation of toxic byproducts, are cause for concern. Alternative disinfectants such as ozone, chlorine

dioxide, and chloramines have similar drawbacks (Kim et al., 2009; Bridier et al., 2011), and the quenching of these disinfectants to prevent membrane damage can lead to the regrowth of bacteria in the RO process. Several biological approaches for the control of biofouling have been investigated (e.g., quorum sensing inhibition, energy uncoupling, and enzyme treatments) (Yeon et al., 2009; Kappachery et al., 2010; Xiong and Liu, 2010; Kim et al., 2011; Xu and Liu, 2011; Yu et al., 2012), but these methods are generally not broadly effective and are only applicable on a narrow spectrum of microorganism strains.

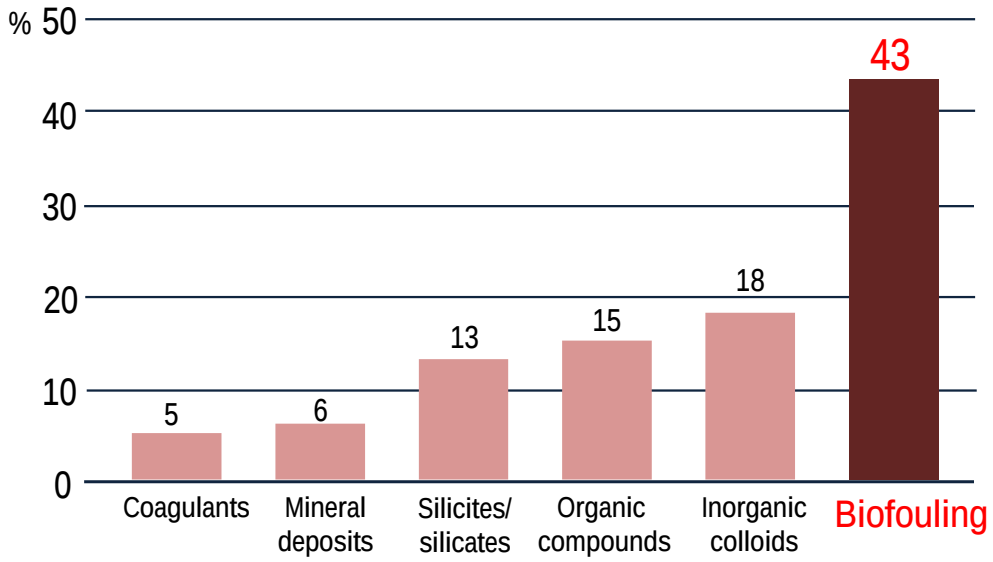

Figure 1. 2 *Major components of membrane fouling*

There are two major approaches to minimize the occurrence of biofouling in the RO membrane process. Firstly, pretreatment is carried out to inactivate microorganisms before the RO membrane process. However, a few microorganisms that successfully survived in the pretreatment step can form biofilm on the RO membrane surface. For this reason, clean-in-place (CIP) treatment is routinely conducted to recover permeate flux, as well as reduction of operation pressure by the inactivation of biofilm cells. In this dissertation, these two aspects of approach for biofouling control were discussed for copper-based disinfection systems.

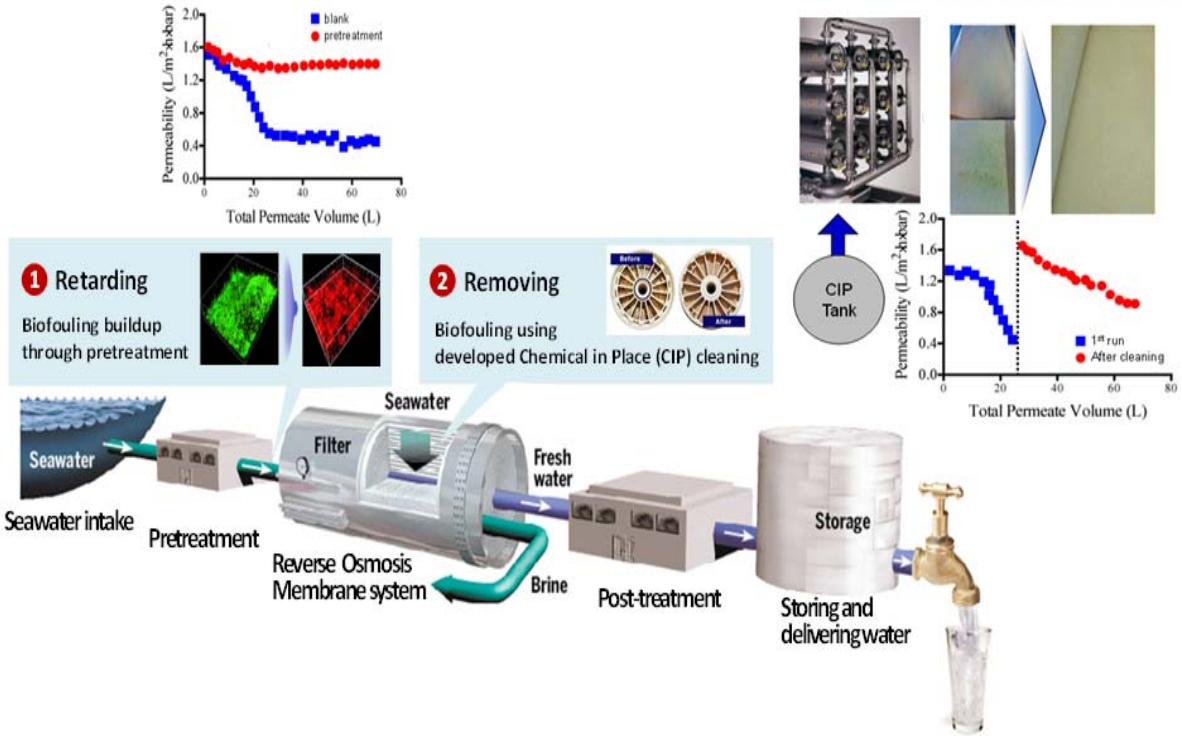

Figure 1. 3 *Major approaches for biofouling control*

1.2 Background and related researches

Copper has been recognized as a useful disinfectant and antimicrobial agent for inactivation of various microorganisms (Borkow and Gabbay, 2004; Dwidjosiswojo et al., 2011). Several studies have demonstrated the successful inactivation of biofilms by using copper, although the inactivation efficiency for the biofilms was not as high as for planktonic cells (Teitzel and Parsek, 2003; Harrison et al., 2005; Baker et al., 2010). It has been suggested that the biocidal action of Cu(II) primarily results from the reduction of Cu(II) to the more toxic Cu(I) species by cellular components (Beswick et al., 1976; Park et al., 2012).

1.2.1 Combination of cupric ion with hydroxylamine and hydrogen peroxide for the control of bacterial biofilms on RO membranes

Recently, we demonstrated that biocidal activity of Cu(II) can be greatly enhanced by the addition of H_2O_2 or hydroxylamine (HA), which accelerates the production of Cu(I) and also generates a reactive oxidant (cupryl ion, Cu(III)) capable of exerting oxidative cell damages (Nguyen et al., 2013; Kim et al., 2015). The Cu(II)/H_2O_2 system was more selective to inactivate viruses than bacteria by favoring the production of Cu(III), believed to be a strong virucidal species (Nguyen et al., 2013), whereas the Cu(II)/HA system non-selectively inactivates both bacteria and viruses by dual microbicidal effects of Cu(I) and Cu(III) (Kim et al., 2015). It is anticipated that the external addition of H_2O_2 into the Cu(II)/HA system (Cu(II)/HA/H_2O_2) further enhances the production of Cu(III).

Despite substantial literature detailing the antimicrobial activity of copper compounds and related systems, reports of using these copper-based disinfection systems for biofilm control are rare. To date, there are only a few studies on the use of metallic copper and the Cu(II)/H_2O_2 system for biofilm inactivation (Wood et al., 1996; Santo et al., 2008), and the Cu(II)/HA and Cu(II)/HA/H_2O_2 systems have not been applied to biofilm cells. Several studies also have reported that surface modifications of RO membranes with copper hydroxide and copper nanoparticles can reduce biofouling (Ben-Sasson et al., 2014; Ben-Sasson et al., 2016; Karkhanechi et al., 2013). However, little is known about the control of membrane biofouling using externally-supplied copper ion and its combined disinfectants. Since the Cu(II)/HA and Cu(II)/HA/H_2O_2 systems simultaneously produce two microbicidal species of different property (i.e., Cu(I) and Cu(III)) (Kim et al., 2015), it would be interesting to observe how these species play roles in the inactivation of biofilm cells. Moreover, tests of biofouling control on RO membranes will provide insight into feasibility of these disinfection systems in the RO desalination process.

1.2.2 Biofilm control on RO membrane by the copper ion in combination with norspermidine

Inactivation of biofilm has presented a challenge for decades, due to the extracellular polymer substances (EPSs), which increases resistance to oxidative disinfectants and biocides (Bridier et al., 2011). In particular, oxidative disinfectants, including chlorine, chlorine dioxide, chloramine, and ozone are effective in the planktonic state, but their efficacy largely decreases in biofilm inactivation, (Kim et al., 2008) as penetration of the disinfectants to reach the inner part of biofilm is delayed by the multiple layers of cells and the EPS. Transport of oxidant-based disinfectant into biofilm is often retarded by inferior penetration caused by the high capacity for reaction with the constituents of biofilm (Stewart et al., 2001). In addition, this often produces undesirable toxic disinfection byproducts. On the other hand, biocide shows moderate or mild resistance to biofilm compared with oxidative disinfectants (e.g., biofilm inactivation of biocide such as silver shows only 28-40 times more resistant to disinfectants than planktonic cells, while chlorine was 8,300-10,000 times more resistant for *E. coli* and *P. aeruginonsa*) (Kim et al., 2008).

Biofilm dispersal in aged biofilms naturally occurs by signal molecules that are self-produced to dissemble in the life cycle of biofilm as the biofilm matures (Vlamakis et al., 2013). Recently, signal (small) molecules, such as D-amino acids and polyamine, have been demonstrated to be applicable to triggering or mediating biofilm disassembly with the inhibition of biofilm formation (Kolodkin-Gal et al., 2010; Si et al., 2014; Yu et al., 2012; Yu et al., 2016). In addition, this technique was applied to mitigate membrane biofouling by promoting biofilm detachment (Xu and Liu, 2011; Yu et al., 2012).

Norspermidine (Nspd), a kind of polyamine, is a small organic hydrocarbon that is positively charged at physiological pH. It plays a role as a biofilm disruptor by binding to negatively charged groups, such as the exopolysaccharide component of biofilm (Böttcher et al., 2013; Oppenheimer-shaanan et al., 2013). Recent publications have shown that Nspd can be pervasive to biofilm at millimolar concentrations, and prevent biofilms formation by a diverse range of bacterial species, including *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Salmonella enterica* and *P. aeruginosa* (Böttcher et al., 2013; Nesse et al., 2015; Qu et al., 2016). Some studies have been conducted on the disassembly effect and mechanism of Nspd (Si et al., 2014; Nesse et al., 2015; Qu et al., 2016; Wu et al., 2016), but Nspd has not been studied for application to membrane biofouling. Despite the impressive performance of Nspd for biofilm disposal, only a few studies have been conducted. In addition, no study is available on the control of biofilm on RO membrane surface by adapting Nspd, though a few studies have been conducted using D-amino acid (Xu and Liu, 2011; Yu et al., 2012).

Copper has been reported as an effective biocide for planktonic cells, as well as biofilms (Teitzel et al., 2003; Harrision et al., 2005; Lee et al., 2017). Lately, the use of Cu(II) ion associated with HA as

a reductant have shown outstanding inactivation efficacy on *E. coli*, and confirmed that Cu(I) is more toxic than Cu(II) (Park et al., 2012; Kim et al., 2015). We demonstrated the biocidal activity of Cu(II), and the combination (Cu(II)/H_2O_2, Cu(II)/HA, Cu(II)/HA/H_2O_2) for inactivation of biofilm formed on membrane (Lee et al., 2017). We found penetration of biofilm into the internal layers, which hampered their efficacy. Therefore, it is expected that addition of Nspd can enhance the inactivation efficacy of the copper-based system on biofilms by the collapse of the EPS.

1.2.3 Inactivation of planktonic and biofilm cells by Cu(II)-activated persulfate in the presence of chloride ion

Lately, oxidation technologies using persulfate (i.e., peroxymonosulfate (PMS), and peroxydisulfate (PDS)) is considered as an emerging oxidant for water treatment and soil remediation by generating sulfate radical anion ($SO_4^{\bullet-}$), which is a highly reactive oxidant ($E^0(SO_4^{\bullet-}/ SO_4^{2-})$ = 2.43 V_{NHE}) (Tsitonaki et al., 2010; Huie et al., 1991). Activation of persulfates to produce $SO_4^{\bullet-}$ is commonly induced by thermal activation (Huang et al., 2002; Waldemer et al., 2007; Liang and Bruell, 2008, Liang and Su, 2009), UV photolysis (Dogliott and Hayon, 1967), alkali activation (Furman et al., 2010), and transition metal catalysis (House, 1962; Thompson, 1981; Furholz and Haim, 1987; Gilbert et al., 1988; Anipsitakis and Dionysiou, 2004). Furthermore, recent studies have suggested some novel ways to activate persulfate, such as carbonnanotubes (CNTs), noble metal nanoparticles, and iodide ions (Lee et al., 2015; Ahn et al., 2016; Feng et al., 2017).

Chemical oxidants including ozone, chlorine, hydrogen peroxide, and ferrate, have been extensively studied as effective tools to degrade the recalcitrant organic contaminants in water; but they have also long been applied for water disinfectants, due to the biocidal effect induced by reactive oxidants. In addition, ozone and a few chlorine-based oxidants are currently being used in drinking water treatment plants. Various ranges of microorganism, such as spores of *Bacillus atrophaeus*, *Bacillus thuringiensis*, *Aspergillus niger*, and *E. coli*, were successfully inactivated by utilizing PMS, although related studies for inactivation are quite limited, compare to the numerous works involving persulfate for the degradation of organic contaminants in wastewater or groundwater (Anipsitakis et al., 2008; Delcomyn et al., 2006). The Cobalt/PMS system was also shown to be an effective disinfection reagent (Anipsitakis et al., 2008). Besides, PMS is widely applied in swimming pool and spas to maintain the concentration of active chlorine (Cl_2) and bromine (Br_2) without production of toxic byproduct. PMS is a solid type, which makes it easier for shipping and more manageable. Furthermore, in situ chlorine can be produced via the reaction of PMS and chloride ion. This will be beneficial for the disinfection of seawater, since seawater contains the large amounts of sodium chloride. In previous study, PMS and sodium chloride in bicarbonate-buffered aqueous solution was confirmed as an

alternative disinfectant for the inactivation of various microorganisms (Delcomyn et al., 2006). However, there is no study available to control biofilms on membrane.

Copper is known as an antimicrobial agent. In particular, the combination of Cu(II) and hydrogen peroxide has shown high inactivation efficiency for bacteria and viruses (Nguyen et al., 2013). In addition, it was found to also be effective disinfectants for the control of bacterial biofilms on RO membranes (Lee et al., 2017). Such combination of oxidizing disinfectants with metallic biocides generally appears to enhance the efficacy of oxidants for disinfection. There are some studies showing the capability of the copper-activated PMS reaction for the production of reactive species, though it was demonstrated using the bimetallic catalyst of copper and iron oxide, acetate buffered medium, and photo-assisted system (Fernandez et al., 2004; Kumar et al., 2012; Ding et al., 2013; Lei et al., 2015; Feng et al., 2016).

1.3 Objective and scopes of the research

The objective of this dissertation is to evaluate three different types of copper-based disinfection systems for the control of biofilm formed on RO membrane surface.

The first part of this dissertation examines the bactericidal effects of Cu(II) and its combined systems with HA and H_2O_2 (i.e., Cu(II)/HA, Cu(II)/H_2O_2, and Cu(II)/HA/H_2O_2) on *P. aeruginosa* cells. Differences in the extent of cell inactivation and the dependence on the state of bacterial cells (planktonic or biofilm) are investigated. In addition, the inactivation of biofilms on RO membranes is tested under different reactor conditions.

The second part of this dissertation investigates the inactivation efficacy of biofilm on RO membranes by the copper ion in combination with Nspd, and elucidates the role of Nspd and the inactivation mechanism. In addition, the biofilm inactivation test was conducted in a cross-flow filtration unit for practical implication.

The third part of this dissertation assesses the potential of chloride ion-enhanced persulfate activation using Cu(II) ion (i.e., PMS, Cu(II), Cu(II)/PMS, PMS/Cl^-, and Cu(II)/PMS/Cl^- systems) for the control of biofilm on RO membranes. The biocidal effects of these treatments were examined for planktonic cells and biofilms formed on the membrane surface. The mechanism through which the inactivation is induced by the treatment is discussed based on the obtained results, focusing on the produced reactive oxidants by combination with PMS. The effect of the Cu(II)/PMS treatment on the RO membrane performance is evaluated in cross-flow filtration units by monitoring the cell concentration, permeate flux, and salt rejection.

II. COMBINATION OF CUPRIC ION WITH HYDROXYLAMINE AND HYDROGEN PEROXIDE FOR THE CONTROL OF BACTERIAL BIOFILMS ON RO MEMBRANES

2.1 Materials and methods

2.1.1 Reagents

Microorganism culture reagents, including tryptic soy broth (TSB) and tryptic soy agar (TSA), were obtained from Becton-Dickinson Co. (USA). Chemicals including copper sulfate ($CuSO_4$), hydrogen peroxide (H_2O_2), hydroxylamine (NH_2OH), sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$), sodium chloride (NaCl), ethylenediaminetetraacetic acid (EDTA), 2,9-dimethyl-1,10-phenanthroline (DMP), 2,4-dinitrophenyl hydrazine (DNPH) and methanol were purchased from Sigma-Aldrich Co. (USA). All chemicals were of reagent grade and used without further purification. All solutions were prepared in deionized water (18 MΩ·cm, Milli-Q water, Millipore Co., USA). Glassware was washed with deionized water and sterilized by autoclave at 121°C for 15 min prior to use.

2.1.2 Culture and analysis of *P. aeruginosa*

P. aeruginosa (ATCC 700829) was incubated in 30 mL of 10% of TSB medium at 37°C for 18 to 24 h. The cells were harvested by centrifugation at 3000 g for 15 minutes and washed three times with phosphate-buffered saline (PBS, pH 7.2) to remove all retained nutrients. The obtained *P. aeruginosa* cells were resuspended in 20 mL of PBS and kept in the refrigerator at 4 to 5°C. The spread plate method was employed to determine the population of *P. aeruginosa*. The number of colonies was counted after incubation of the cells plated on TSA for 24 h at 37°C.

2.1.3 Inactivation experiments of planktonic cells

All inactivation experiments were performed in 60 mL Pyrex reactors at room temperature (22±2°C). *P. aeruginosa* cells were suspended in DI water (pH 7.0) at the initial population of approximately 10^7 CFU/mL. The suspensions were then mixed vigorously by magnetic stirrers. The experiment was initiated by adding Cu(II) and auxiliary reagents (HA or H_2O_2) at predetermined concentrations. One-milliliter of sample was taken at predetermined time intervals and immediately quenched with ethylenediaminetetraacetic acid (EDTA) and sodium sulfite. The sample was diluted with PBS to the required population of microbes, and each diluted sample was assayed on triplicate

agar plates. The *P. aeruginosa* inactivation was expressed as $\log(N/N_0)$, in which N_0 and N indicate the initial number of cells and the number of cells in treated sample, respectively.

2.1.4 Analyses of formaldehyde and Cu(I)

The production of formaldehyde (HCHO) (from the oxidation of excess methanol) and Cu(I) was monitored in combined systems of Cu(II) with HA and H_2O_2. HCHO production in the presence of excess methanol is indicative of the generation of reactive oxidants (i.e. Cu(III)). HCHO was analyzed by HPLC with UV absorbance detection at 350 nm after the DNPH derivatization (Zhou and Mopper, 1990). Cu(I) was analyzed by the neocuproine method (American Public Health Association et al., 2005).

2.1.5 Culture and inactivation of biofilms in CDC reactors

Biofilms of *P. aeruginosa* were grown in center for disease control (CDC) biofilm reactors (CBR 90, Biosurface Technologies Co., USA). One reactor contains eight rods, and each rod holds three glass coupons (borosilicate glass coupons with diameter 1.27 cm) for sampling. On these coupons, RO membranes (LFC3-LD; Hydranautics a Nitto Denko, USA) of 1 cm × 1 cm were attached with 3M double-sided tape. *P. aeruginosa* cells (10^6 CFU/mL) were initially inoculated in the CDC reactor containing 350 mL of 1% TSB culture medium. The reactor was operated in a batch mode for 24 h at room temperature (22±2°C) in 1% TSB medium while stirring at 100 rpm. After 24 h of batch culture, 0.3% TSB medium was continuously flowed through the CDC reactor at a flow rate of 11.67 mL/min for 24 h. Then, each rod was withdrawn from the reactor, and gently rinsed with deionized water to remove the TSB solution left on coupons (taking care not to disturb the formed biofilms).

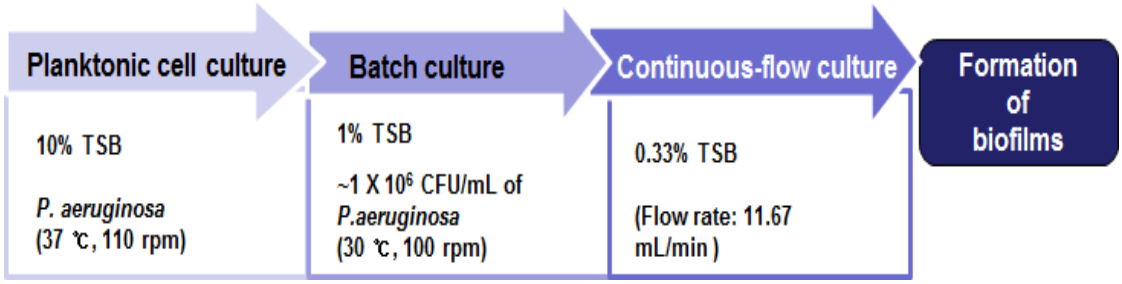

Figure 2. 1 *Protocol for biofilm formation in CDC reactor.*

Inactivation experiments were conducted in a 350 mL bottle equipped with rods of biofilm coupons. The experiment was initiated by adding reagents (Cu(II), HA or H_2O_2) with stirring at 100 rpm, and individual rods were removed at different sampling times. Cells and EPS in the biofilm matrix were detached from the coupons by ultrasonication for 3 min and vortexing for 3 min in 10 ml PBS solution. The number of cells was counted by a spread plate method.

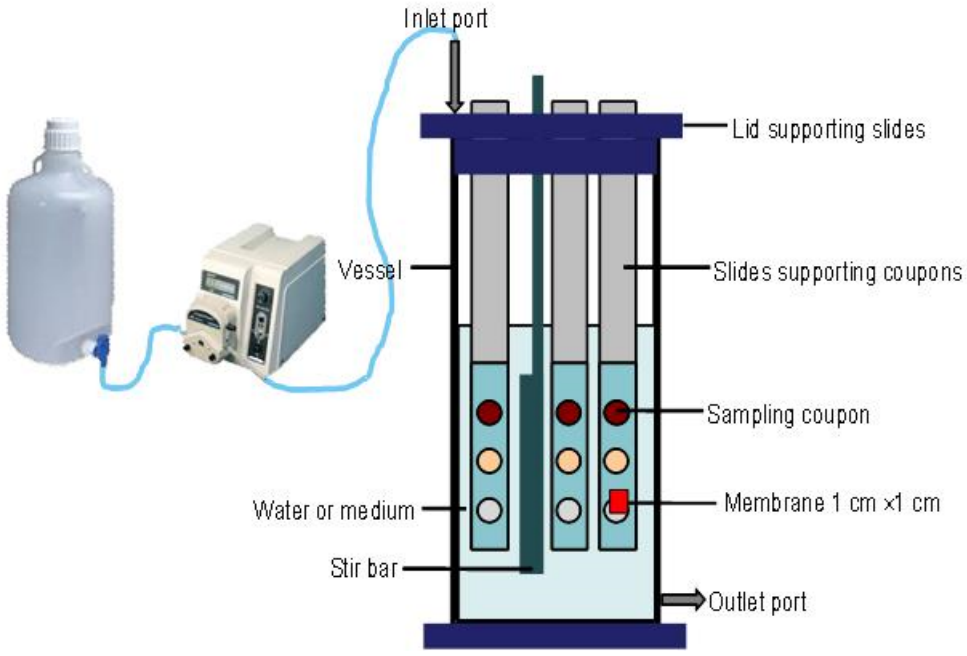

Figure 2. 2 *Center for disease control (CDC) biofilm reactor, cross-sectional view.*

2.1.6 Experiments in a cross-flow filtration unit

Biofouling experiments were carried out in a lab-scale cross-flow filtration unit (Fig. 2. 3) with a commercial RO membrane sample (LFC3-LD; Hydranautics a Nitto Denko, USA) of 4 cm × 6 cm flat sheet. The solution in the feed tank was continuously filtered by the RO membrane at constant pressure, and after the filtration the concentrate and the permeate were mixed into the feed tank for recirculation.

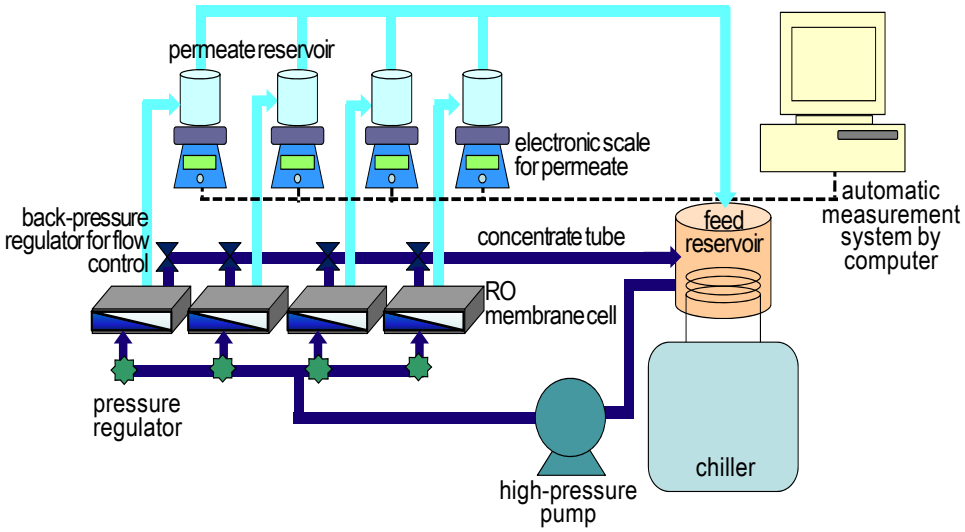

Figure 2. 3 *Schematic diagram of a lab-scale cross-flow filtration unit equipped with RO membranes.*

Prior to the experiment, the cross-flow filtration unit was thoroughly disinfected and cleaned by the sequential recirculation of 1% sodium hypochlorite, 5 mM EDTA at pH 11, and 2 mM sodium dodecyl sulfate (SDS), and each step was conducted at a flow rate of 1 L/min for 1 h (Herzberg and Elimelech, 2007). Membrane samples were installed into the system, compacted for 18 h operation with deionized water, and conditioned with feed solution containing 10 mM NaCl, 10 mM sodium citrate, and 0.1% tryptic soy broth for 6 h (Baek et al., 2011). Operating conditions were as follows: initial flux = 55 L/m^2/h, cross-flow velocity = 12.8 cm/s, pressure = 15.5 bar, and temperature = 22±2°C. To grow biofilms on membrane samples, 10^7 CFU/ml *P. aeruginosa* was inoculated into the feed solution, and the system was operated under the conditions described above. The permeate flux was continuously monitored from the point of *P. aeruginosa* inoculation by the automatic measurement setup of permeate weight. Salt rejection (i.e., Salt rejection (%) = 100 × (1 − C_p/C_f)) was calculated by measuring the conductivity of feed (C_f) and the permeate (C_p) using a conductivity meter (Ultrameter II, Myron L Co., USA).

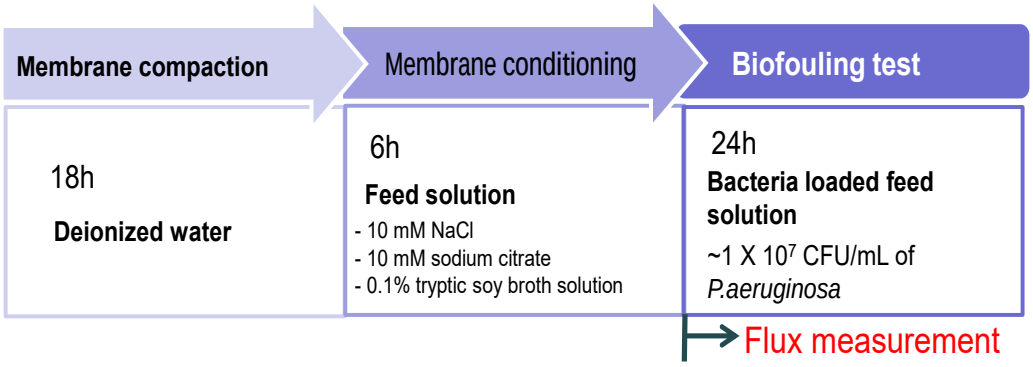

Figure 2. 4 *Protocol for biofilm formation in a cross-flow filtration unit.*

To examine the biofilm inactivation by Cu(II)-based disinfection systems, disinfectants were applied after 24 h of biofilm growth. The biofilm inactivation experiment was initiated by injecting reagents (Cu(II), HA or H_2O_2) into the feed water tank. RO membrane samples were carefully taken at predetermined time intervals and washed with deionized water. Bacterial cells were detached from the RO membrane in the same manner as described for the inactivation of bacterial biofilms in CDC reactors and were analyzed by a spread plate method. In addition, the biofilms formed on membrane surfaces were visualized by a confocal laser scanning microscope (CLSM; FV1000, Olympus Co., Japan) with Imaris software (Bitplane Co., Switzerland) after staining with the BacLight Live/Dead bacterial viability kit (Molecular Probes Co., USA). After 4 h of the treatment, the feed solution was replaced with fresh solution containing 10 mM NaCl to remove planktonic cells grown in the tank. Then, the permeate flux and salt rejection were monitored for another 12 h to evaluate the cleaning of RO membranes by disinfectants.

2.2 Results and discussion

2.2.1 Inactivation of planktonic cells by Cu(II)-based disinfection systems

The inactivation of *P. aeruginosa* by Cu(II), Cu(II)/HA, Cu(II)/H_2O_2, and Cu(II)/HA/H_2O_2 systems was examined on planktonic cells (Fig. 2. 5). H_2O_2, HA, and their mixture (HA/H_2O_2) without Cu(II) had negligible effects on the *P. aeruginosa* inactivation (Fig. 2. 6). Cu(II) alone did not significantly inactivate *P. aeruginosa*. In contrast, combined systems showed enhanced inactivation of planktonic *P. aeruginosa*, and the inactivation efficacies for the systems were Cu(II)/H_2O_2 (1.7 log in 30 min) < Cu(II)/HA (5 log in 10 min) < Cu(II)/HA/H_2O_2 (5 log in 5 min).

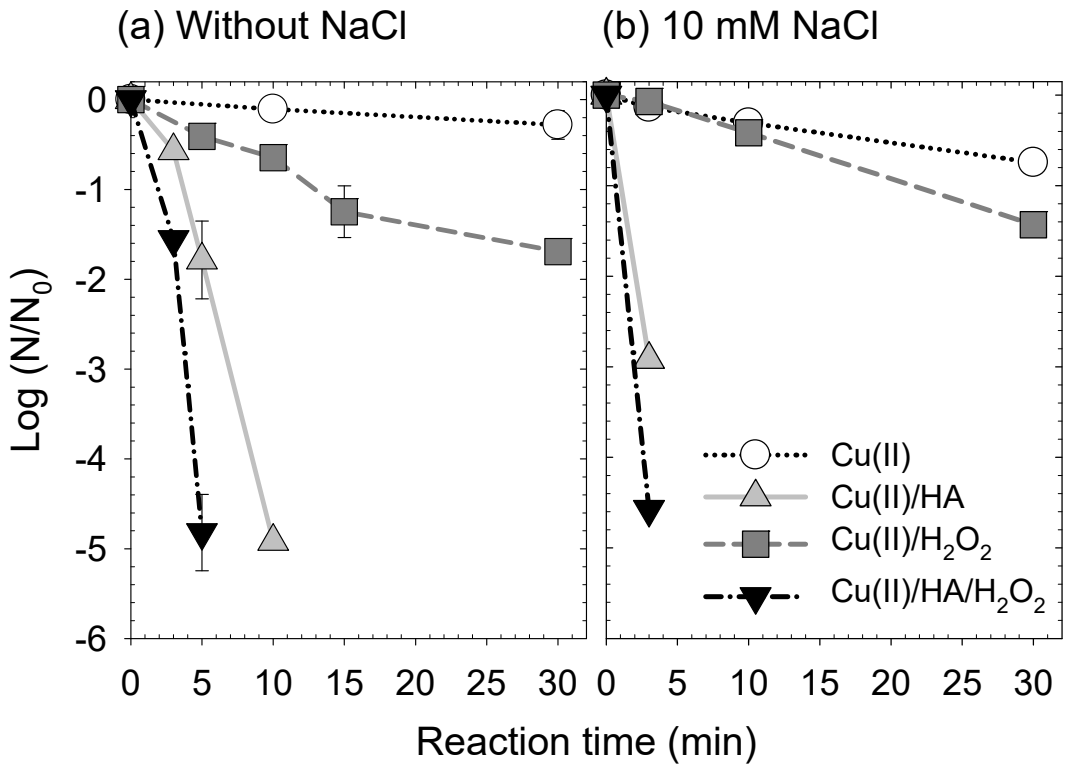

Figure 2. 5 *Inactivation of planktonic P. aeruginosa cells by different Cu(II)-based disinfection systems in the (a) absence and (b) presence of NaCl (Initial cell concentration: ~1 × 10^7 CFU/mL, pH = 7.0, $[Cu(II)]_0$ = 5 μM, $[H_2O_2]_0$ = 0.1 mM, $[HA]_0$ = 0.1 mM, $[NaCl]_0$ = 10 mM for (b)).*

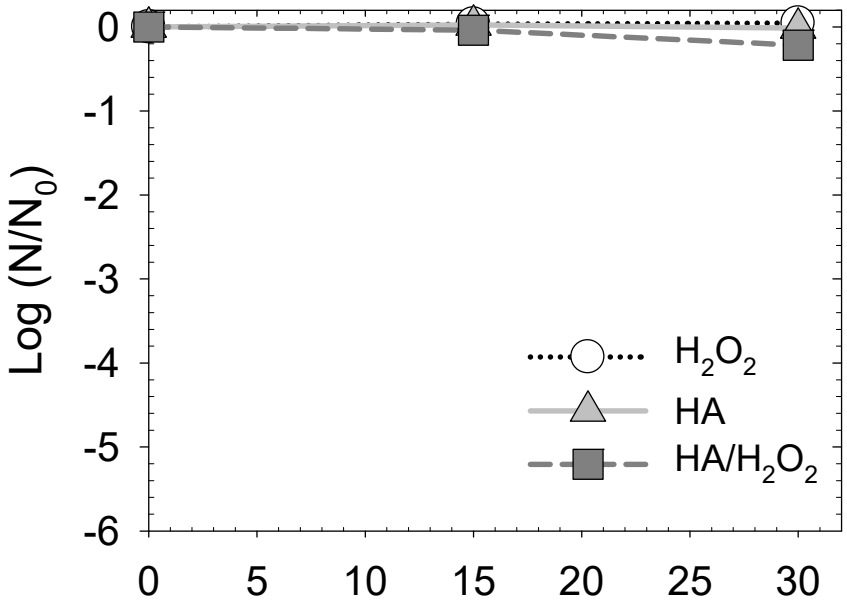

Figure 2. 6 *Inactivation of planktonic P. aeruginosa cells by H_2O_2, HA, and their mixture (HA/H_2O_2) (Initial cell concentration: ~1 × 10^7 CFU/mL, pH = 7.0, $[Cu(II)]_0$ = 5 μM, $[H_2O_2]_0$ = 0.1 mM, $[HA]_0$ = 0.1 mM).*

The bactericidal activity of Cu(II)/H_2O_2, Cu(II)/HA, and Cu(II)/HA/H_2O_2 systems mainly result from the dual biocidal effects by Cu(I) and Cu(III) (as described in reactions 1–6) (Nguyen et al., 2013; Kim et al., 2015). The Cu(II)/H_2O_2 system produces Cu(I) and Cu(III) via Fenton-like reactions (reactions 1 and 2) and exhibits improved inactivation efficacies for both bacteria and viruses compared to Cu(II) alone. In particular, the biocidal action of Cu(III) was found to be more selective for viruses.

$$Cu(II) + H_2O_2 \rightarrow Cu(I) + O_2^{\bullet-} + 2H^+ \quad (1)$$

$$Cu(I) + H_2O_2 \rightarrow Cu(III) + 2OH^- \quad (2)$$

The Cu(II)/HA system is also known to inactivate microorganisms by dual mechanisms (i.e., biocidal actions of Cu(I) and Cu(III)) (Kim et al., 2015). First, HA acts as a reducing agent, accelerating the conversion of Cu(II) into the more toxic Cu(I) (reaction 3).

$$2NH_2OH\ (HA) + 4Cu(II) \rightarrow N_2O + H_2O + 4Cu(I) + 4H^+ \quad (3)$$

Additionally, consecutive reactions of Cu(I) with O_2 and $O_2^{\bullet-}$ (reactions 4 and 5) and the reaction of HA with O_2 (reaction 6) can both produce H_2O_2 in situ, which subsequently generates Cu(III) (reaction 2).

$$Cu(I) + O_2 \rightarrow Cu(II) + O_2^{\bullet-} \quad (4)$$

$$Cu(I) + O_2^{\bullet-} + 2H+ \rightarrow Cu(II) + H_2O_2 \quad (5)$$

$$2NH_2OH + O_2 \rightarrow N_2 + H_2O_2 + 2H_2O \quad (6)$$

The production of HCHO (the oxidation product of methanol by Cu(III)) and Cu(I) was monitored in each system (Fig. 2. 7). Comparison of the Cu(II)/HA and Cu(II)/H_2O_2 systems revealed that the Cu(II)/HA system exhibited greater production of both HCHO and Cu(I) (Fig. 2. 7). This result indicates that the higher inactivation efficacy of the Cu(II)/HA system over the Cu(II)/H_2O_2 system (Fig. 2. 5a) is caused by an increase in concentration of both Cu(I) and Cu(III). Meanwhile, the Cu(II)/HA/H_2O_2 system resulted in even greater bacterial inactivation than the Cu(II)/HA system (Fig. 2. 5a). According

to the chemistry described in reaction 1–6, the external addition of H_2O_2 into the Cu(II)/HA system enhances the production of Cu(I) and Cu(III) by accelerating the Fenton-like reactions (reactions 1 and 2) (positive effect). However, because reaction 2 is faster than reaction 1, the consumption of Cu(I) can be more pronounced (negative effect). The superior activity of the Cu(II)/HA/H_2O_2 system compared to the Cu(II)/HA system indicates that the positive effect prevails in this trade-off mechanism. Indeed, the HCHO production by the Cu(II)/HA/H_2O_2 system was much larger than in the Cu(II)/HA system, whereas the difference in Cu(I) concentration was relatively small (Fig. 2. 7).

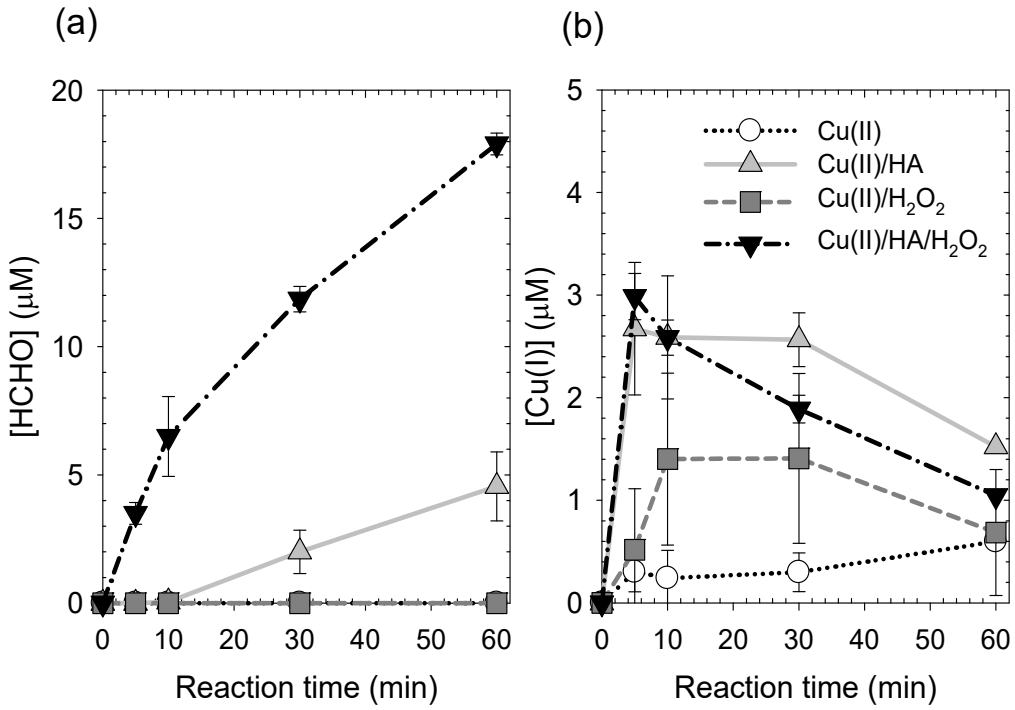

Figure 2. 7 *Production of (a) HCHO and (b) Cu(I) by different Cu(II)-based disinfection systems ($[Cu(II)]_0$ = 5 μM, $[H_2O_2]_0$ = 0.1 mM, $[HA]_0$ = 0.1 mM, $[Methanol]_0$ = 200 mM for (a), pH = 7.0).*

The inactivation of *P. aeruginosa* by Cu(II)-based disinfection systems under anoxic conditions (N_2 conditions) showed similar trends to those under oxic conditions (Fig. 2. 8). The Cu(II)/HA and Cu(II)/HA/H_2O_2 systems exhibited slightly enhanced bacterial inactivation under anoxic conditions, indicating that Cu(I) may be more bactericidal than Cu(III); anoxic conditions increase the level of Cu(I) concentration by preventing the oxidative consumption of Cu(I) by oxygen and inhibiting the production of Cu(III). The higher bactericidal effect under anoxic conditions has also been reported for the *E. coli* inactivation by the Cu(II)/HA system (Kim et al., 2015).

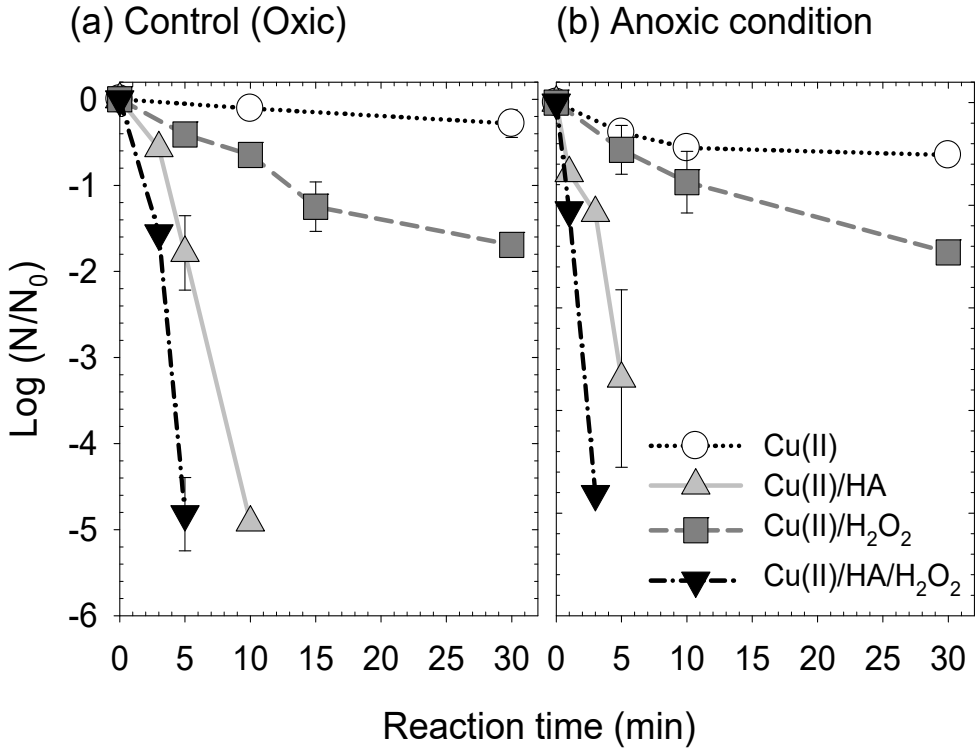

Figure 2. 8 *Inactivation of planktonic P. aeruginosa cells by different Cu(II)-based system under (a) oxic and (b) anoxic conditions (Initial cell concentration: ~1 × 10^7 CFU/mL, pH = 7.0, $[Cu(II)]_0$ = 5 μM, $[H_2O_2]_0$ = 0.1 mM, $[HA]_0$ = 0.1 mM).*

The effect of NaCl on the *P. aeruginosa* inactivation by different Cu(II)-based disinfection systems was examined (Fig. 2. 5). It appears that overall trends are not significantly affected by the addition of NaCl (compare Figs. 2. 5a and b). However, Cu(II)/HA and Cu(II)/HA/H_2O_2 systems showed noticeable enhancements in bacterial inactivation efficacy; the Cu(II)/HA and Cu(II)/HA/H_2O_2 systems with NaCl achieved 2.9 and 4.5 log inactivation in 3 min, respectively. The effect of NaCl can be explained by the speciation change of Cu(II) from $Cu(OH)_2$ into $CuCl_2$ (refer to Fig. 2. 9). Standard redox potentials of copper complexes positively increase as Cu(II) forms complexes with chloride ($E^0(Cu^{2+}/Cu^+) = 0.153\ V_{NHE}$, $E^0(CuCl^+/CuCl^0) = 0.330\ V_{NHE}$, $E^0(CuCl_2^0/CuCl_2^-) = 0.567\ V_{NHE}$) (Moffett and Zika, 1987), which facilitates the reductive conversion of Cu(II) into Cu(I) by reducing agents. The accelerated conversion of Cu(II) into Cu(I) not only increases the steady-state concentration of Cu(I) but also promotes the generation of Cu(III) mediated by the reaction of Cu(I) with H_2O_2 (reaction 2). This enhancement of biocidal activity in the presence of NaCl encourages application of these disinfection systems in seawater.

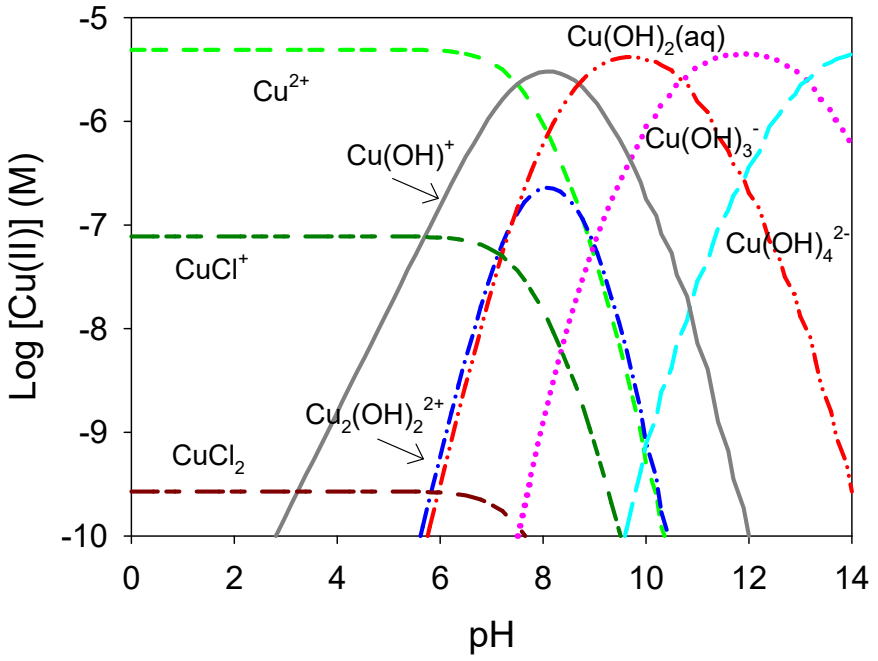

Figure 2. 9 *Speciation of Cu(II) in the presence of 10 mM NaCl ($[Cu(II)]_0$ = 5 μM, $[NaCl]_0$ = 10 mM; calculated using MINEQL+ with the stability constants provided in the database).*

2.2.2 Inactivation of biofilms on RO membranes by Cu(II)-based disinfection systems

The inactivation of *P. aeruginosa* cells in biofilms by Cu(II)-based disinfection systems was examined in CDC reactors (Fig. 2. 10a). The initial population of cells in biofilms grown on the RO membrane was approximately 10^9 CFU/cm^2 before the treatment. Overall, biofilm cells were more resistant to the disinfection systems than planktonic cells. For biofilm cells, much higher doses of reagents were required to achieve comparable levels of inactivation: $[Cu(II)]_0$ = 0.1 mM; $[H_2O_2]_0$ = 1 mM; $[HA]_0$ = 1 mM (compare to the reagent doses in Fig. 1). In addition, the inactivation efficiency order of the systems toward biofilm cells was Cu(II)/HA < Cu(II) < Cu(II)/HA/H_2O_2 < Cu(II)/H_2O_2 (Fig. 2. 10a), in sharp contrast to the inactivation of planktonic cells (Fig. 2. 5a). This order did not change at lower doses of Cu(II) (Fig. 2. 11).

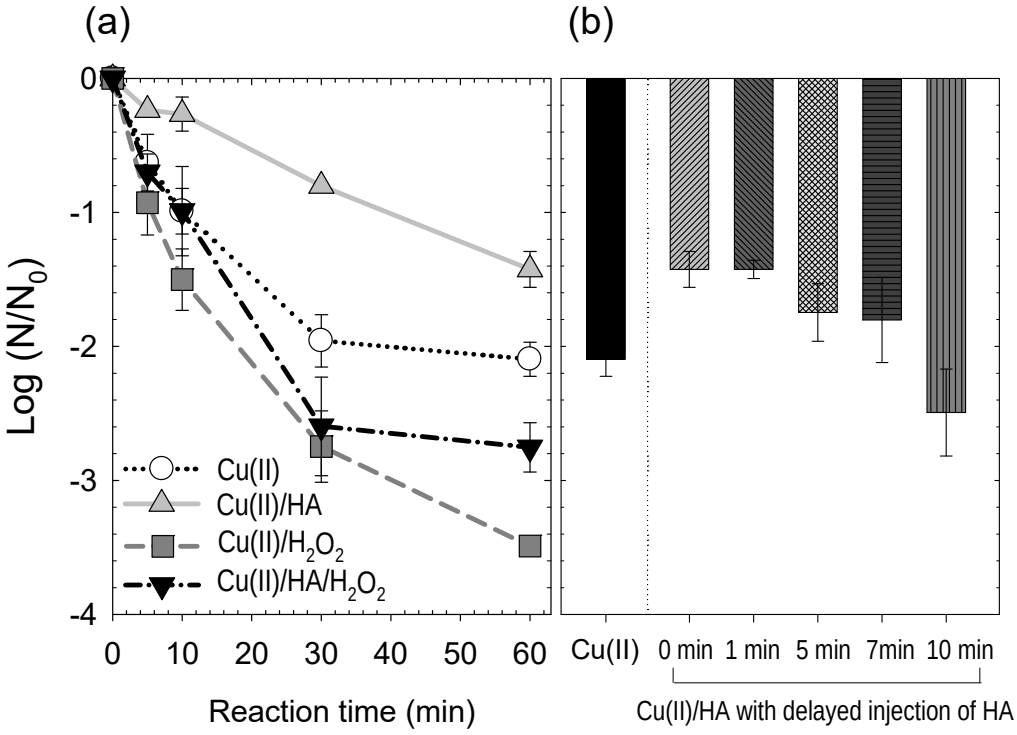

Figure 2. 10 *(a) Inactivation of P. aeruginosa cells in biofilms by different Cu(II)-based disinfection systems, and (b) effects of delayed injections of HA on the inactivation of biofilm cells by the Cu(II)/HA system (Initial cell concentration: ~1 × 10^9 CFU/cm^2, pH = 7.0, $[Cu(II)]_0$ = 0.1 mM, $[H_2O_2]_0$ = 1 mM, $[HA]_0$ = 1 mM, reaction time = 60 min for (b)).*

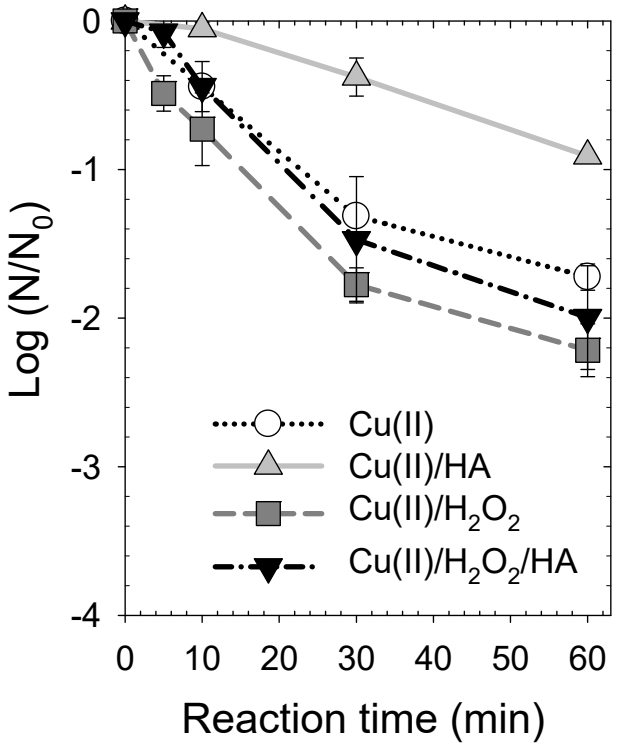

Figure 2. 11 *Inactivation of P. aeruginosa cells in biofilms by different Cu(II)-based disinfection systems of lower Cu(II) doses ($[Cu(II)]_0$ = 0.05 and 0.1 mM, $[H_2O_2]_0$ = 1 mM, $[HA]_0$ = 1 mM, pH = 7.0).*

By considering Cu(II)/HA < Cu(II) and Cu(II)/HA/H_2O_2 < Cu(II)/H_2O_2, it appears the addition of HA inhibits the biocidal activity of Cu(II) in the biofilm matrix. Because the primary role of HA is to reduce Cu(II) to Cu(I), the data suggests that Cu(I) is less useful in the inactivation of cells in biofilms than the Cu(II) form. This is unexpected because, as was mentioned previous, Cu(I) is known to be more biocidal than Cu(II), and the cytotoxicity of Cu(II) primarily results from the Cu(I) produced by cellular reduction of Cu(II). A possible explanation for this behavior is that Cu(I) may not penetrate into the cells in biofilms as readily as Cu(II). It is presumed that Cu(I) is more selectively blocked by the EPS barrier than Cu(II), preventing it from reaching the cell. Indeed, the copper content in biofilms treated by Cu(II) was higher than that treated by the Cu(II)/HA system (Fig. 2. 12).

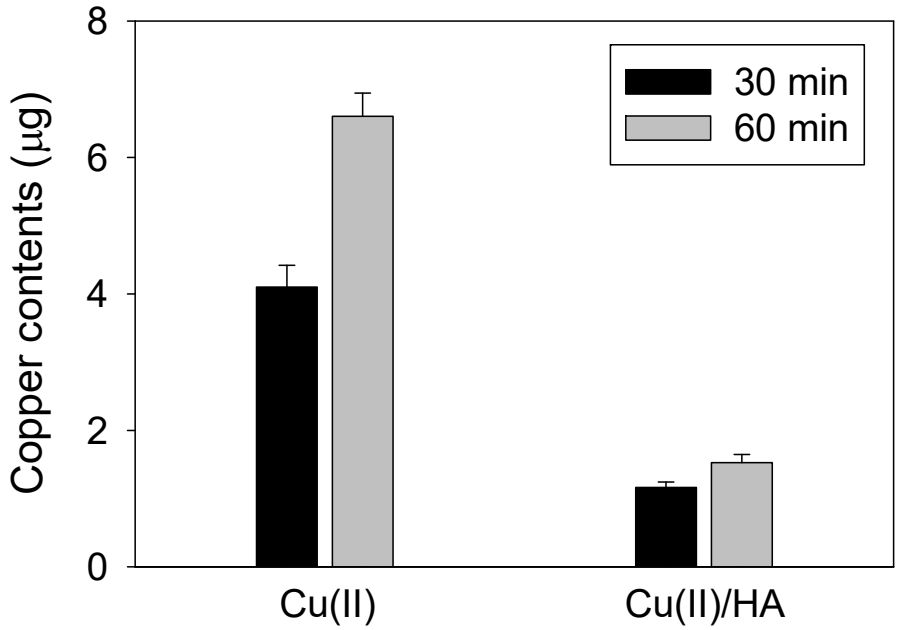

Figure 2. 12 *Copper contents in biofilms treated by Cu(II) and Cu(II)/HA. ($[Cu(II)]_0$ = 0.1 mM, $[H_2O_2]_0$ = 1 mM, $[HA]_0$ = 1 mM, pH = 7.0; the mass of total copper in biofilms on a RO membrane sample (1 cm × 1 cm) was measured).*

The limited accessibility of Cu(I) to cells in biofilms was further proved by a delayed injection of HA in the Cu(II)/HA system (i.e., the reaction was initiated by adding Cu(II) first, then after several minutes HA was injected). This delayed injection allowed for Cu(II) to diffuse into biofilms. As shown in Fig. 2. 10b, the cell inactivation efficacy of the Cu(II)/HA system increased as the delay time of HA injection increased. In particular, the Cu(II)/HA system with 10 min delayed injection of HA exhibited a higher inactivation efficacy than Cu(II) alone, indicating that the conversion of Cu(II) to Cu(I) improves the inactivation of biofilm cells if sufficient time is given for Cu(II) to contact the cells.

Finally, cell inactivation experiments were performed using physically disrupted biofilms. RO membrane samples with biofilms were subjected to sonication and vortexing (for 3 min each) in a PBS solution to obtain the suspension of cells and disrupted EPS. Then, the inactivation of cells in the suspension was examined by treating with different Cu(II)-based disinfection systems (Fig. 2. 13). As a result, bacterial cells in the suspension of disrupted biofilms were more rapidly inactivated overall (compare Figs. 2. 13a and 13b). More importantly, the inactivation efficacy in Fig. 2. 13b increased in the order of Cu(II) < Cu(II)/H_2O_2 < Cu(II)/HA < Cu(II)/HA/H_2O_2, similar to the case of planktonic cells (Fig. 2. 5a). These observations collectively indicate that the structural integrity of biofilms significantly contributes to the reduced penetration of Cu(II)-based disinfectants to the biofilm matrix, which is in agreement with previous reports on the importance of intact biofilm structure to resist Cu(II) biocides (Gonzalez et al., 2010; Fang et al., 2011).

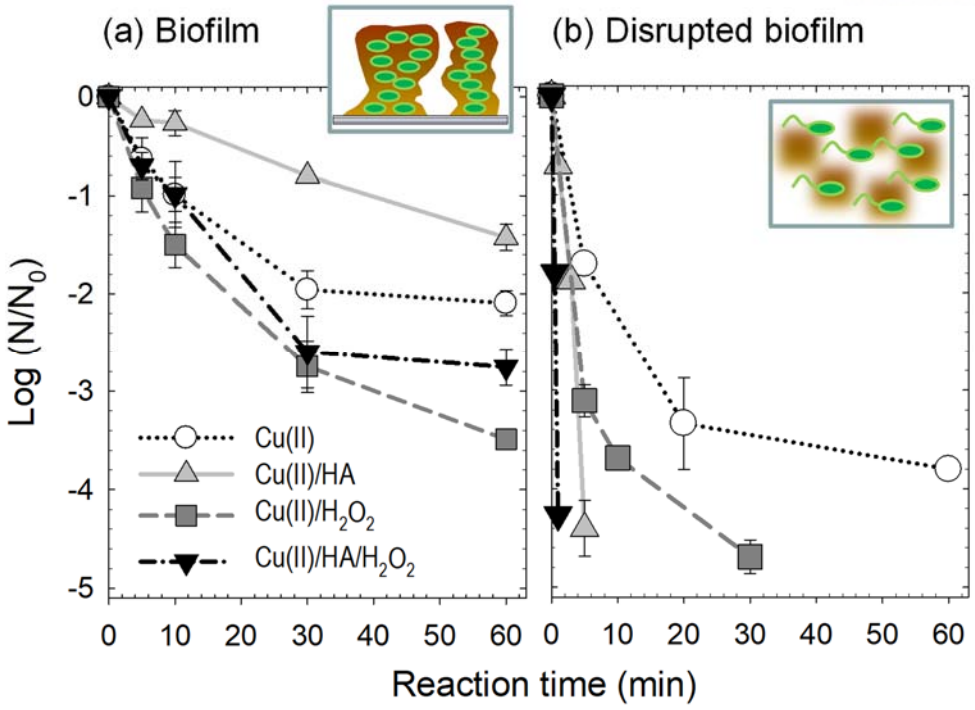

Figure 2. 13 *Inactivation of P. aeruginosa cells in (a) intact and (b) disrupted biofilms by different Cu(II)-based disinfection systems ($[Cu(II)]_0$ = 0.1 mM, $[H_2O_2]_0$ = 1 mM, $[HA]_0$ = 1 mM, pH = 7.0).*

2.2.3 Inactivation of biofilms on RO membranes in a cross-flow filtration unit

Biofilms were grown on RO membranes in a cross-flow filtration unit and the cell population on RO membranes, the permeate flux, and the salt rejection were monitored for 48 h (Fig. 2. 14). The permeate flux significantly declined as the cell population on the RO membranes increased (biofilms formed on the RO membrane surface), and the salt rejection marginally decreased from 99.8% into 99.6%. In contrast, the control experiment without cells showed minimal decrease in the permeate flux.

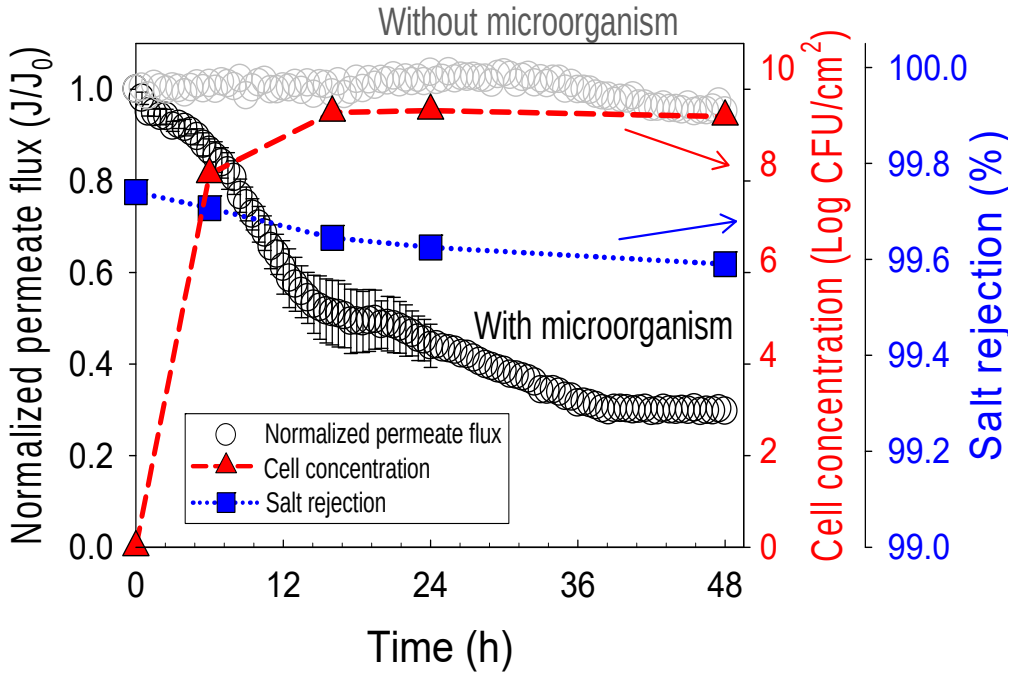

Figure 2. 14 *Variations in normalized permeate flux, salt rejection, and bacterial cell concentration in biofilms during the biofilm growth in a cross-flow filtration unit (Tested membrane = LFC3-LD, Initial cell concentration in feed solution = ~1 × 10^7 CFU/mL, Initial permeate flux = 55 L/m^2/h, Applied pressure = 15.5 bar, pH = 6.0).*

To determine the extent of biofilm inactivation by different Cu(II)-based disinfection systems, disinfectants (Cu(II), Cu(II)/HA, Cu(II)/H_2O_2 and Cu(II)/HA/H_2O_2) were applied after 24 h of biofilm growth (Fig. 2. 15). The Cu(II) and Cu(II)/H_2O_2 treatments did not inactivate the biofilm cells at all (Figs. 2. 15a and 15b). The Cu(II)/HA treatment showed a small cell inactivation from approximately 10^9 CFU/cm^2 to $10^{8.7}$ CFU/cm^2, which corresponds to 50% cell inactivation. (refer to the inset of Fig. 2. 15c). The Cu(II)/HA/H_2O_2 treatment exhibited a significant degree of the cell inactivation (6 log inactivation in 4 h) (Fig. 2. 15d).

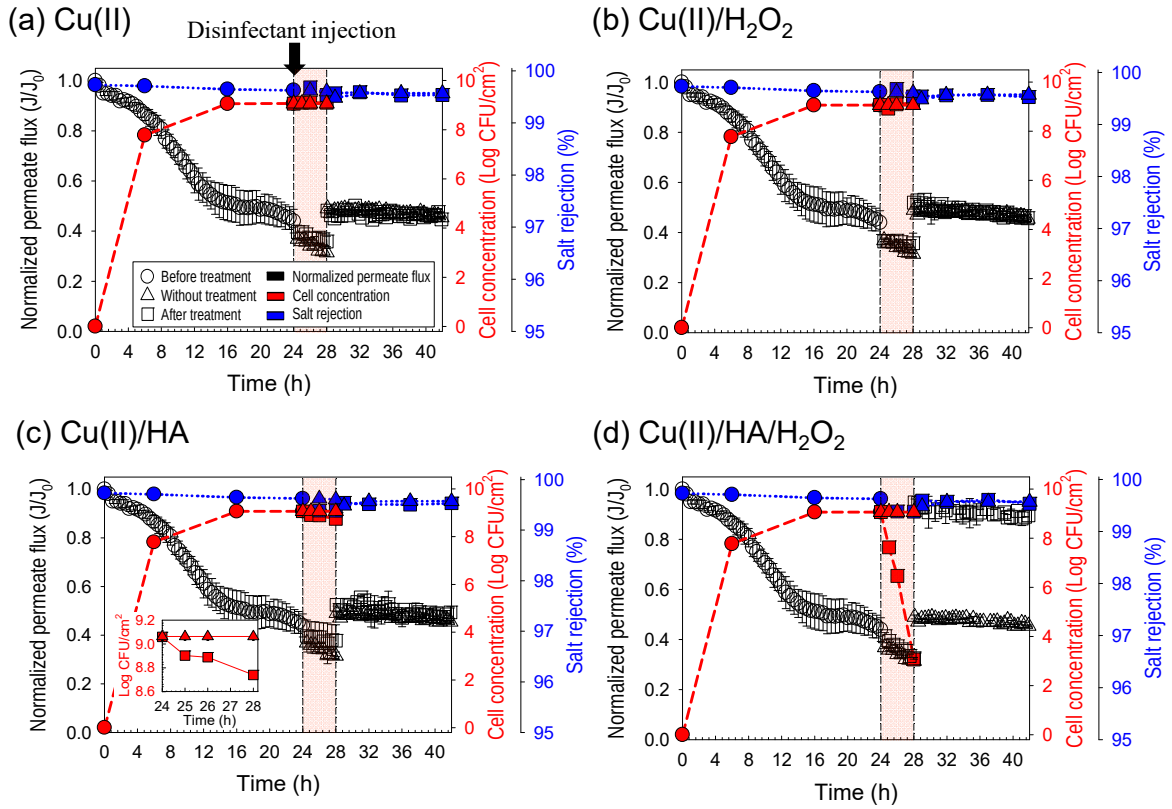

Figure 2. 15 *Variations in normalized permeate flux, salt rejection, and bacterial cell concentration in biofilms in a cross-flow filtration unit: biofilms were grown for 24 h, and subsequently disinfectants were injected (Initial permeate flux = 55 L/m^2/h, Applied pressure = 15.5 bar, pH = 6.0, $[Cu(II)]_0$ = 0.1 mM, $[H_2O_2]_0$ = 1 mM, $[HA]_0$ = 1 mM, $[NaCl]_0$ = 10 mM).*

CLSM images show that cells in biofilms are mostly dead (indicated by cell membrane damage) after two hours of exposure to the Cu(II)/HA/H_2O_2 system, and after 4 h of treatment most of the dead cells are destructed (Fig. 2. 16). Overall, the efficacies of Cu(II)-based disinfection systems appear to be lowered in the cross-flow filtration unit compared to the CDC reactor. Because biofilms in the cross-flow filtration unit are formed by compression under high pressure (15.5 bar), EPS barriers are densified and are more resistant to disinfectants. Several previous studies also demonstrated that the structure of biofilms formed on RO membranes under the hydraulic pressure is compact and dense, as observed by CLSM, scanning electron microscopy (SEM), and hydrodynamic resistance of biofilms (Dreszer et al., 2013; Herzberg and Elimelech, 2007; Herzberg et al., 2009; Kwan et al., 2015). Meanwhile, the permeate flux was recovered by more than 90% after the Cu(II)/HA/H_2O_2 treatment due to the significant inactivation of biofilms (Fig. 2. 15d). However, the flux recovery by the other treatments (Cu(II), Cu(II)/HA and Cu(II)/H_2O_2) was negligible (Figs. 2. 15a–15c).

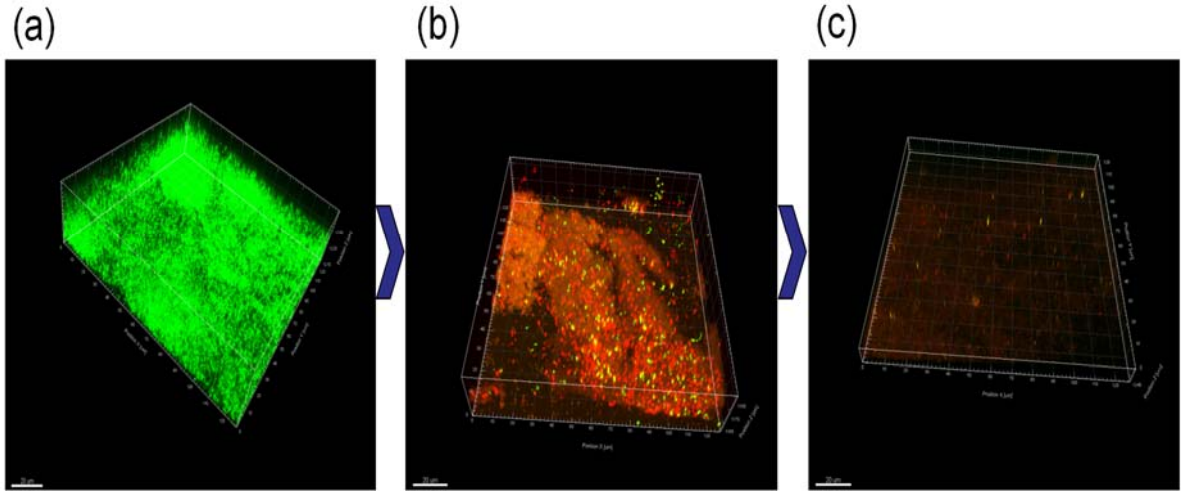

Figure 2. 16 *CLSM images of Cu(II)/HA/H_2O_2-treated P. aeruginosa biofilms on RO membranes in a cross-flow filtration unit: (a) before treatment (24 h biofilm growth), (b) after 2 h treatment and (c) after 4 h treatment (scale: 126.7 μm × 126.7 μm × 15−20 μm, live and dead cells are green and red, respectively).*

In the cross-flow filtration unit, it is notable that the disinfection treatments with HA (i.e., Cu(II)/HA and Cu(II)/HA/H_2O_2) work better than those without HA for the inactivation of biofilm cells in the cross-flow filtration unit, similar to the case of planktonic cells (Fig. 2. 5a). It should be noted that the inactivation efficacy of biofilm cells in CDC reactors increased in the order of Cu(II)/HA < Cu(II) < Cu(II)/HA/H_2O_2 < Cu(II)/H_2O_2 (Fig. 2. 10a) because Cu(I) has limited cell accessibility in the EPS matrix (refer to the previous section). A possible explanation is that the penetration of Cu(I) into the biofilm matrix is facilitated due to the presence of membrane permeate flux, improving the exposure of Cu(I) to cells.

2.2.4 Effects of pretreatments by Cu(II)-based disinfection systems on biofilm growth

The feed solution containing *P. aeruginosa* cells was initially treated by different disinfection systems, and the permeate flux decline and the biofilm cell growth on RO membranes was monitored over time (Fig. 2. 17). Treatment with Cu(II) alone (Fig. 2. 17a) showed similar biofouling to the control with no disinfectant (Fig. 2. 14). The Cu(II)/H_2O_2 treatment inhibited the cell growth, impeding the decline in the permeate flux in the early stage (Fig. 2. 17b). However, the flux decline was accelerated after 24 h as the cell population steadily increased. The Cu(II)/HA treatment was more effective in suppressing the regrowth of cells. The cell population in biofilms remained low for 48 h (approximately 10^2 to 10^4 CFU/mL with fluctuations), which significantly mitigated the flux decline (Fig. 2. 17c). The Cu(II)/HA/H_2O_2 treatment completely blocked the cell regrowth, and the level of flux decline was similar to that of Cu(II)/HA (Fig. 2. 17d). CLSM images of biofilms initially treated by the Cu(II)/HA/H_2O_2 system show that dead cells attached on RO membranes are gradually destructed due to the oxidative disruption by Cu(III) as the exposure time increases (Fig. 2. 18). The cake formation by dead cells and cellular debris appears to be responsible for 25–30% flux decline observed in the Cu(II)/HA and Cu(II)/HA/H_2O_2 treatments.

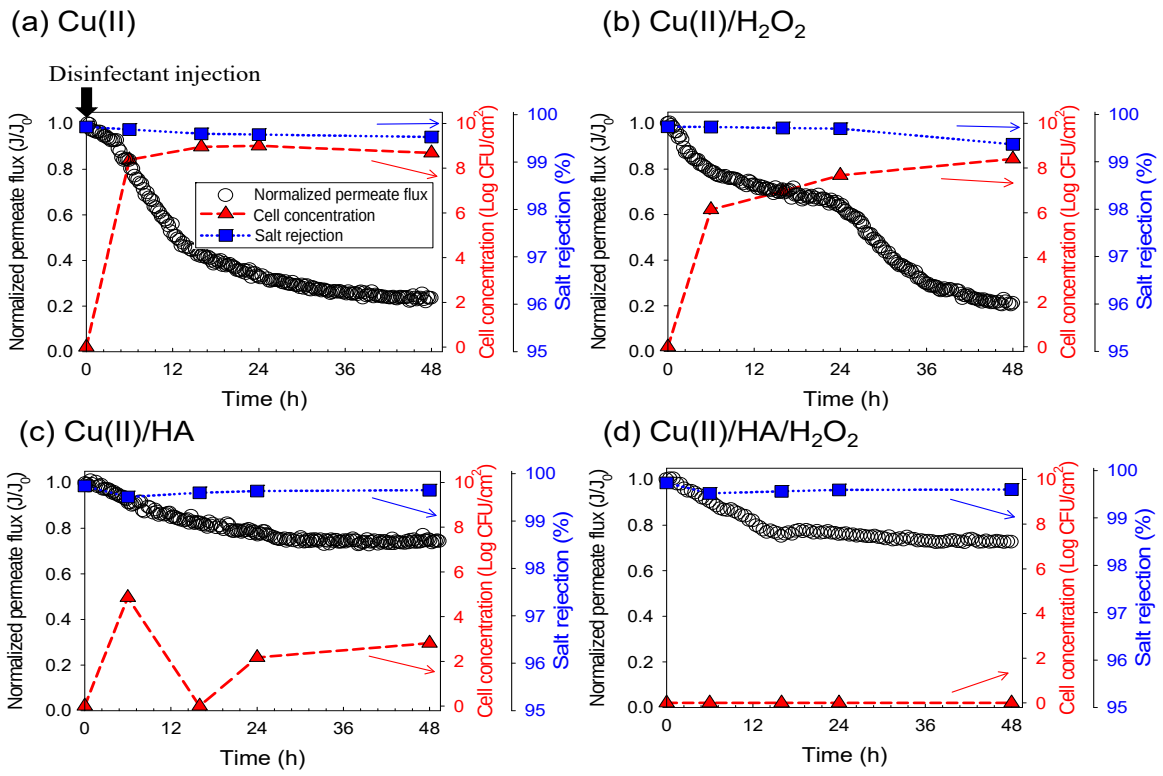

Figure 2. 17 *Variations in normalized permeate flux, salt rejection, and bacterial cell concentration in biofilms in a cross-flow filtration unit: disinfectants were initially applied (Initial permeate flux = 55 L/m^2/h, Applied pressure = 15.5 bar, pH = 6.0, [Cu(II)]$_0$ = 0.1 mM, [H_2O_2]$_0$ = 1 mM, [HA]$_0$ = 1 mM, [NaCl]$_0$ = 10 mM).*

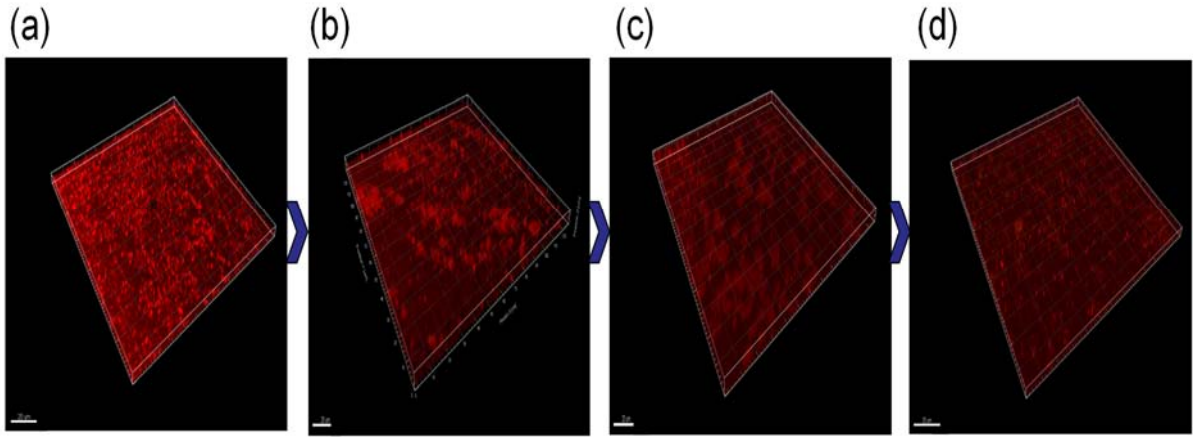

Figure 2. 18 *CLSM images of biofilms initially treated by the Cu(II)/HA/H_2O_2 system in a cross-flow filtration unit: (a) after 6 h, (b) after 16 h, (c) after 24 h, and (d) after 48 h (scale: 126.7 μm × 126.7 μm × 15−20 μm, live and dead cells are green and red, respectively).*

2.3 Summary

The combination of cupric ion with hydroxylamine and hydrogen peroxide was found to be effective for both planktonic and biofilm cells, and shows the potential for the application as cleaning reagent to control biofilms formed on RO membrane. The major conclusions and environmental implications are summarized as follows.

- The Cu(II)-based disinfection systems effectively inactivate bacterial cells in both planktonic and biofilm states. Particularly, the Cu(II)/HA and Cu(II)/HA/H_2O_2 systems are strong disinfectants that rapidly inactivate planktonic bacterial cells with a low dose of copper. Importantly, the use of 5 μM Cu(II) achieved 5 log bacterial inactivation in 10 min for Cu(II)/HA and in 5 min for Cu(II)/HA/H_2O_2 (Fig. 2. 5a), and drinking water quality standards for copper are 1 mg/L (= 15.7 μM) in Korea and 1.3 mg/L (= 20.5 μM) in the United States.
- The Cu(II)/HA and Cu(II)/HA/H_2O_2 systems can be widely applied where proper disinfection is required. Particularly, they can be used as pretreatment disinfectants to control the biofouling of RO membranes in the desalination process as pretreatment with the Cu(II)/HA and Cu(II)/HA/H_2O_2 systems successfully prevented the biofilm growth on RO membranes, mitigating the permeate flux decline (Fig. 2. 17). The enhanced bactericidal activity in the presence of chloride ion (Fig. 2. 5b) will be beneficial for their application in seawater.
- The Cu(II)/HA/H_2O_2 system is also capable of inactivating cells in biofilms on RO membranes in the cross-flow filtration unit (Fig. 2. 15). Its application as a membrane cleaning reagent is possible, as the permeate flux was successfully recovered by the Cu(II)/HA/H_2O_2 treatment (Fig. 2. 15d). However, for the other treatments (Cu(II), Cu(II)/HA and Cu(II)/H_2O_2), alternative methods (e.g., increasing doses or combination with mechanical washing or other reagents) need to be considered for the membrane cleaning application.
- The different performances of Cu(II)-based disinfection systems for the biofilm inactivation depending on the type of reactor (Fig. 2. 10a vs. Fig. 2. 15) imply that the selection of these disinfection systems should be made by considering the hydraulic environment where biofilms are formed and the process employed (e.g., the performance may be different for forward osmosis (FO) versus RO operations). The application of Cu(II)-based disinfection systems appears far-reaching and has implications beyond simply membrane filtration processes.

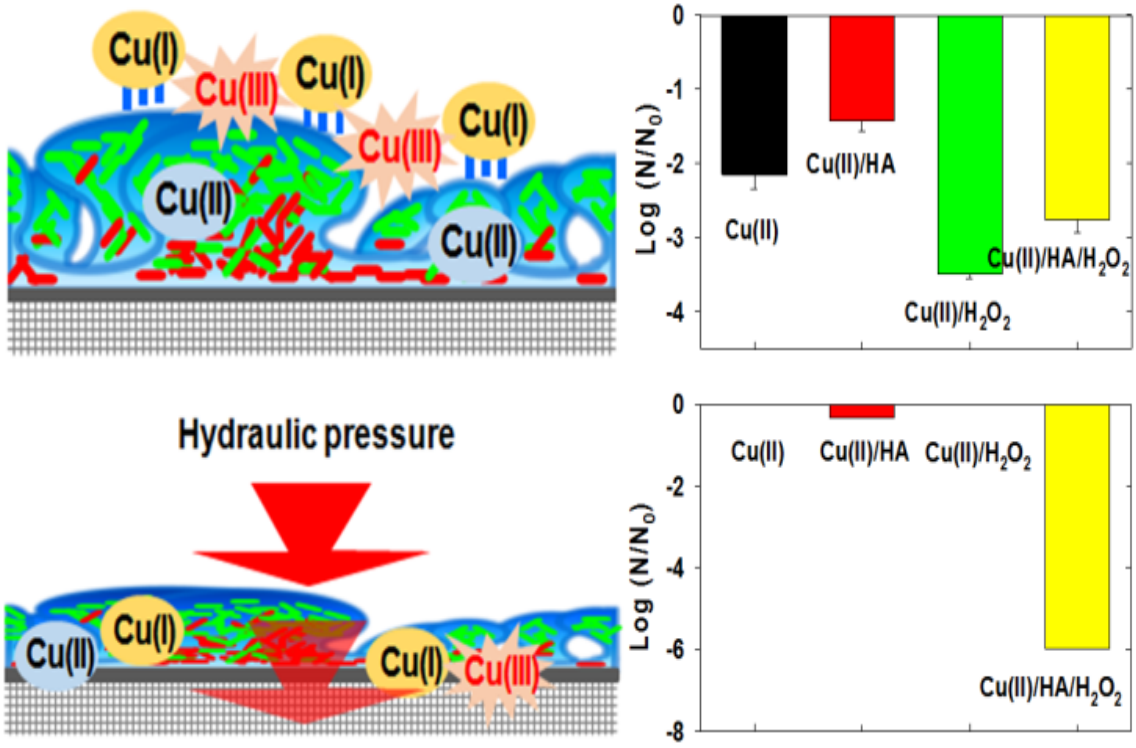

Figure 2. 19 *Scheme for suggested inactivation mechanism by the combination of cupric ion with hydroxylamine and hydrogen peroxide.*

III. BIOFILM CONTROL ON RO MEMBRANE BY THE COPPER ION IN COMBINATION WITH NORSPERMIDINE

3.1 Materials and methods

3.1.1 Reagents

Microorganism culture reagents, including tryptic soy broth (TSB) and tryptic soy agar (TSA), were obtained from Becton-Dickinson Co. (USA). Copper sulfate ($CuSO_4$), norspermidine (Nspd), ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA-Na_2), hydroxylamine (NH_2OH), sodium chloride (NaCl), sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) was purchased from Sigma-Aldrich Co. (USA). All chemicals were of reagent grade and used without additional purification. All glassware was deionized water and sterilized by autoclave at 121 °C for 15 min prior to use.

3.1.2 Culture and analysis of *P. aeruginosa*, and inactivation experiments of planktonic cells

P. aeruginosa was incubated in 30 ml of 10% of TSB medium at 37°C for 18-24 h. The cells were harvested by centrifugation at 3000g for 15 min and washed. All experiments were performed in a batch system at room temperature (22 ± 2°C) using a 60-mL Pyrex reactor open to the atmosphere.

3.1.3 Biofilm in CDC reactors and a cross-flow filtration unit

Biofilms of *P. aeruginosa* were grown in center for disease control (CDC) biofilm reactors (CBR 90, Biosurface Technologies Co., USA). Details of the reactor features and conditions for biofilm growth were described in a previous chapter, and were performed here in the same manner (Lee et al., 2017). Inactivation experiments were conducted in a 350-mL bottle equipped with rods of biofilm coupons. The experiment was initiated by adding the reagents (Cu(II), HA, Nspd) with stirring at 100 rpm, and individual rods were removed at different sampling times. Cells in the biofilm matrix were detached from the coupons by ultrasonication for 3 min and vortexing for 3 min in 10 ml phosphate buffered solution. The number of cells was counted by a spread plate method.

3.1.4 Experiments in a cross-flow filtration unit

Biofouling experiments were carried out in a lab-scale cross-flow filtration unit with a commercial RO membrane sample (LFC3-LD; Hydranautics a Nitto Denko, USA) of 4 cm × 6 cm flat sheet. Detail of cross-flow filtration unit and cleaning procedure can be found in our previous study (Lee et al., 2017).

Cross-flow filtration unit is intended to simulate a spiral wound membrane system for practical application. Membrane samples were installed into the system, and compacted for 18 h operation with deionized water, and conditioned with feed solution containing 10 mM NaCl, 10 mM sodium citrate, and 0.1% tryptic soy broth for 6 h (Baek et al., 2011). Operating conditions were as follows: initial flux = 55 L/m^2/h, cross-flow velocity = 12.8 cm/s, pressure = 15.5 bar, and temperature = 22±2°C. To grow biofilms on membrane samples, 10^7 CFU/ml *P. aeruginosa* was inoculated into the feed solution, and the system was operated under the conditions described above.

The biofilm inactivation experiment was initiated by injecting reagents (Cu(II), HA, Nspd) into the feed water tank. RO membrane samples were carefully taken at predetermined time intervals and washed with deionized water. Bacterial cells were detached from the RO membrane in the same manner as described for the inactivation of bacterial biofilms in CDC reactors and were analyzed by a spread plate method. After 4 h treatment of disinfectants in the inactivation of biofilms experiment, feed solution has replaced with solution containing 10 mM NaCl to remove reagents, and we monitored the permeate flux and salt rejection for another 32 h to evaluate chemical cleaning effects on the performance of RO membrane.

3.1.5 Fluorescent stains of total cell, protein and polysaccharides

The total bacterial cell was measured using 4′,6-diamidino-2-phenylindole (DAPI) (Molecular Probes Co., USA). Specifically, one milliliter of biofilm suspension was filtered on a black polycarbonate for 5 min, and placed on a confocal dish. The numbers of total cells were counted using MetaMorph software (Molecular Devices Co., USA).

To evaluate the amount of protein and polysaccharides, membrane coupon was immersed in FITC solution (Molecular Probes Co., USA) to label proteins (0.01 mg·mL^{-1}) for 1 h, then, subsequently stained with Con A (Molecular Probes Co., USA) to label polysaccharide (0.1 mg·mL^{-1}) (Con A in 0.1 M sodium bicarbonate buffer, pH 8.3) and incubated for another 30 min. After each of these staining stages, the samples were washed twice by 0.1 M sodium bicarbonate buffer (pH 8.8) to retain the amine group in a nonprotonated form, and remove the excess staining solution. After staining, the treated membrane samples were immediately visualized by inverted fluorescence microscopy (IX73, Olympus Co., Japan) with FITC and TRITC filters for proteins (stained green) and polysaccharide (stained red), respectively. Images were randomly obtained from more than 5 locations, and the average bioarea (μm^2) was calculated using MetaMorph software (Molecular Devices Co., USA).

3.2 Results and discussion

3.2.1 Enhanced inactivation of *P. aeruginosa* cells in biofilm by the norspermidine in combination with copper ion

The inactivation of biofilm and planktonic *P. aeruginosa* cells was examined by Cu(II) and Cu(II)/HA in combination with Nspd (Fig. 3. 1). In planktonic and biofilm states, 0.1 mM Nspd alone did not exhibit biocidal activity. The literature has also reported that Nspd can inhibit PAO1 planktonic cell growth from 5 mM Nspd, and inhibited the biofilm formation from 0.1 mM, with biofilm eradication appearing from 1 mM (Qu et al., 2016).

In planktonic states, the bactericidal activity of Cu(II) appeared as 2.2 log in 60 min, and that of Cu(II)/Nspd was suppressed to 1.3 log in 60 min (Fig. 3. 1a). The parallel results in Fig. 3. 1a show between Cu(II)/HA (4.2 log in 5 min) and Cu(II)/HA/Nspd (2.0 log in 10 min). Retarded inactivation efficacy by Cu(II) and Cu(II)/HA in combination with Nspd could be derived by the complexation of copper ion, and it appears lower toxicity of copper ion to planktonic *P. aeruginosa* cells as the polyamine including Nspd can act as a ligand with copper ion (Lomozik and Gasowska, 1998; Perkowska and Maluszynska, 1999; Guskos et al., 2011). This result is expected from the literature that the toxicity of copper ion is reduced by the addition of EDTA (Flemming and Trevors, 1989; James et al., 1998; Ma et al., 2003).

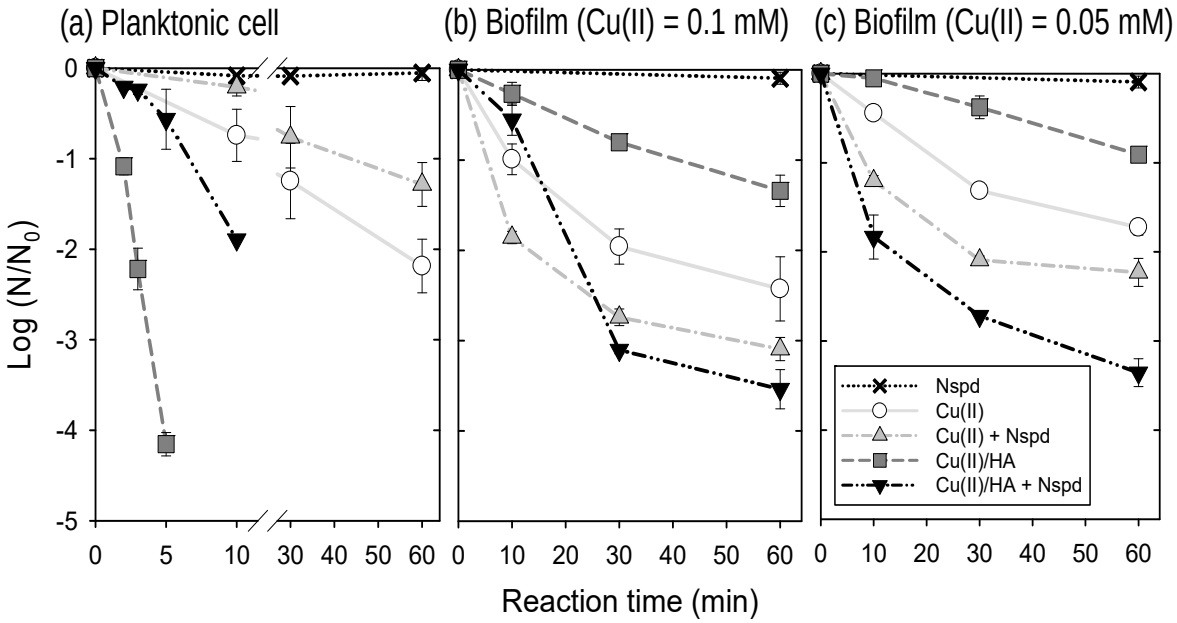

Figure 3. 1 *Effects of Nspd with Cu(II) and Cu(II)/HA on inactivation of planktonic P. aeruginosa cells (a) and P. aeruginosa cells in biofilm (b, c) (Initial cell concentration: ~1 × 10^7 CFU/mL for planktonic cell, ~1 × 10^9 CFU/cm^2 for biofilm; $[Nspd]_0$ = 0.1 mM; $[Cu(II)]_0$ = 0.1 mM (a, b), 0.5 mM (c); $[HA]_0$ = 1 mM; 0.1 mM phosphate buffer at pH_0 = 7.0; reaction time = 1 h; 100 rpm).*

In contrast to the inhibitory effect of Nspd on the inactivation of planktonic *P. aeruginosa* cells, biofilm inactivation result showed enhanced inactivation efficacy in the presence of Nspd (e.g. Cu(II) < Cu(II) + Nspd; Cu(II)/HA < Cu(II)/HA + Nspd). It is noticeable that Cu(II)/HA was the most effective for inactivation in planktonic status due to the toxicity of the Cu(I), but it becomes invalid in a biofilm status that is by the EPS barrier (Lee et al., 2017). It is presumed that Cu(I) is more selectively blocked by the EPS barrier than Cu(II), preventing it from reaching the cell. The cytotoxicity of Cu(I) could work properly by the action of Nspd as a disassembly reagent of biofilm. The inactivation efficacy was also enhanced as the Nspd concentration increases (Fig. 3. 2) due to the synergistic effect of the disassembling effect by Nspd and the toxicity of Cu(I) itself. The inactivation efficacy is dramatically increased with increase of Nspd dose up to 100 μM; then the enhancement of biofilm inactivation is smaller from 100 to 500 μM (Fig. 3. 2). It is noteworthy that the inactivation enhancement between Cu(II)/HA and Cu(II)/HA+Nspd was even more obvious with lower concentration of Cu(II) ion (Figs. 3. 1b and c). This result indicates that penetration of Cu(I) is vital for biofilm inactivation.

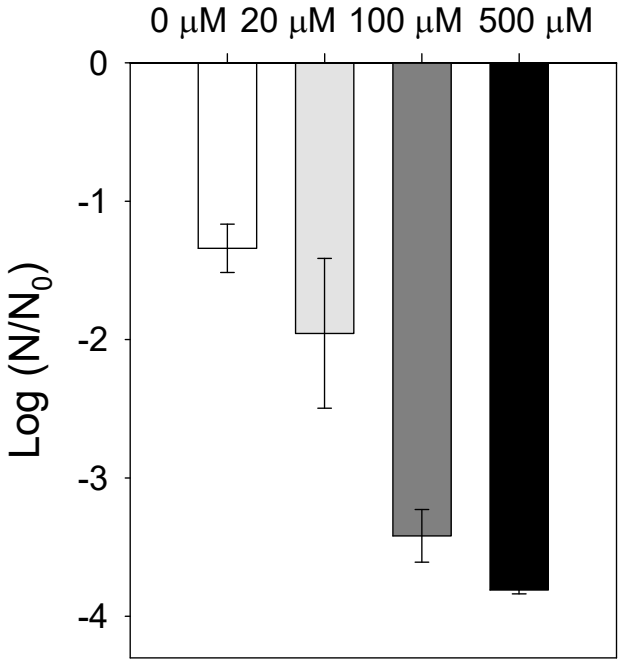

Figure 3. 2 *Inactivation of P. aeruginosa cells in biofilm by the Cu(II)/HA/Nspd system as a function of Nspd concentration (Initial cell concentration: ~1 × 10^9 CFU/cm^2; $[Norspermidine]_0$ = 0.02, 0.1, 0.5 mM; $[Cu(II)]_0$ = 0.1 mM; $[HA]_0$ = 1 mM; 0.1 mM phosphate buffer at pH_0 = 7.0; reaction time = 1 h).*

3.2.2 Effect of norspermidine in combination with copper ion on protein, polysaccharides, and detachment of cells

Disruption of EPS affects biofilm inhibition and eradication, as EPS is responsible for adhesion in biofilm to immobilize biofilm cells and keep them close by allowing cell-cell communication (Flemming and Wingender, 2010). Since Nspd is known as a biofilm disassembly reagent to reduce polysaccharide through the binding with the functional group O-C-O in polysaccharide (Qu et al., 2016; Si et al., 2014), reduction of protein and polysaccharide as a main component of EPS was examined (Fig. 3. 3).

Microscopy images of EPS distribution by each treatment can be found in Fig. 3. 4. The bioarea of polysaccharide was reduced to approximately 50% by Nspd treatment compared to that of protein, while no inactivation occurred under the identical condition (Figs. 3. 3 and 3. 6a). This shows that Nspd is only involved in EPS disruption without the inactivation of cells. Similarly, a recent paper reported that Nspd treatment leads only to polysaccharides reduction with no effect on protein, other than the result that the combined treatment of Nspd and silver causes the reduction of both polysaccharides and protein (Wu et al., 2016).

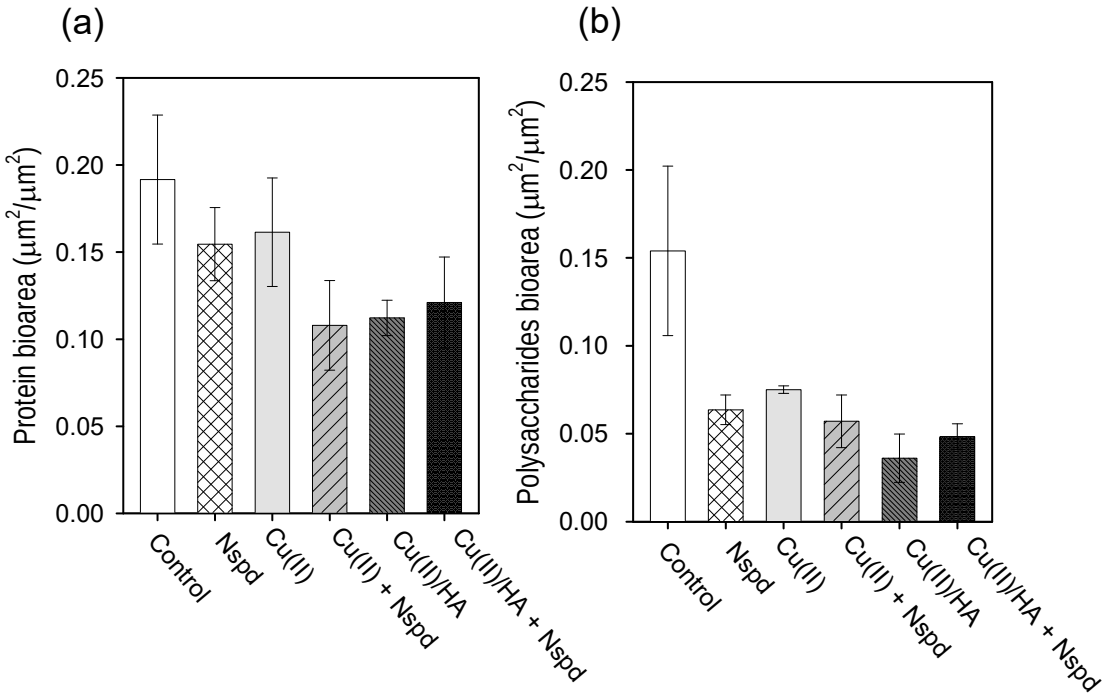

Figure 3. 3 *Bioarea of protein and polysaccharides which calculated from 8−12 randomly sampled images ([Norspermidine]$_0$ = 0.1 mM; [Cu(II)]$_0$ = 0.1 mM; [HA]$_0$ = 1 mM; 0.1 mM phosphate buffer at pH$_0$ = 7.0; reaction time = 1 h; 100 rpm).*

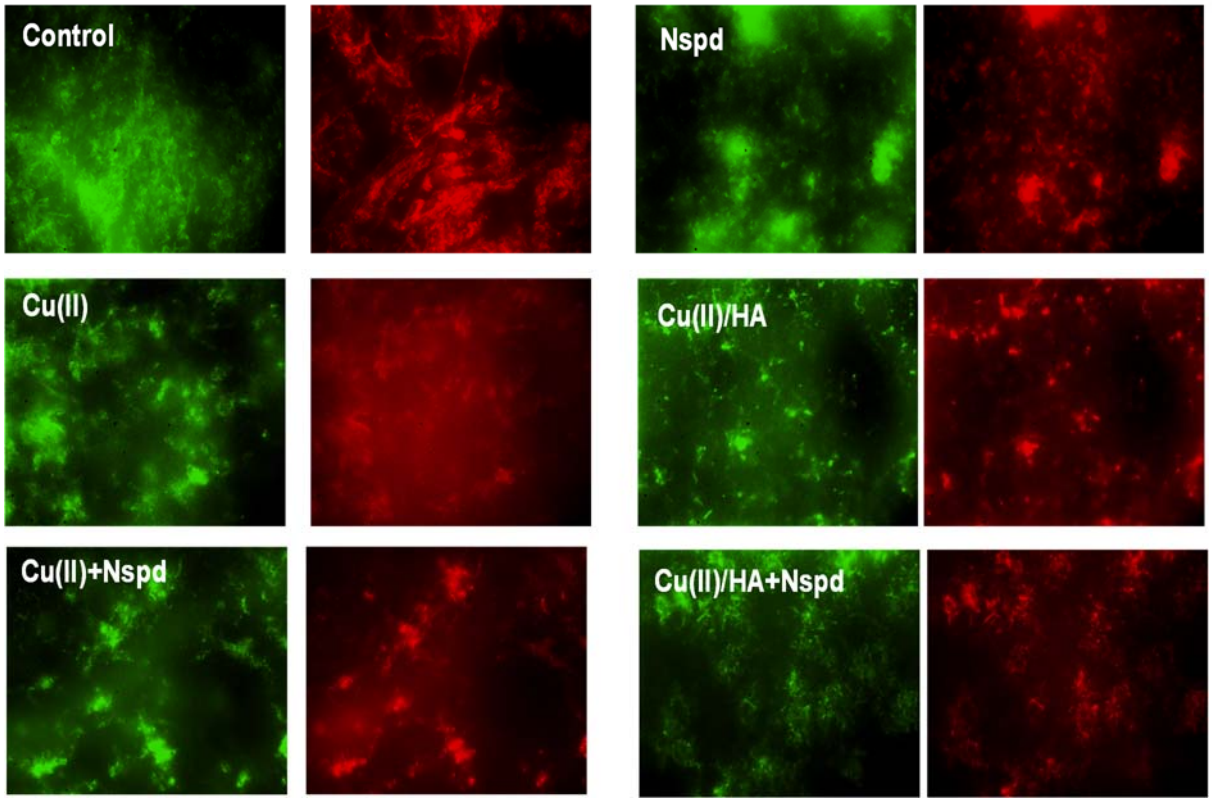

Figure 3. 4 *Microscopy images of EPS distribution by the Cu(II), Cu(II)/HA, Cu(II)/Nspd, and Cu(II)/HA/Nspd treatments. Proteins and polysaccharides were stained green and red with FITC and conA-tetramethylrhodamine, respectively ($[Norspermidine]_0$ = 0.1 mM; $[Cu(II)]_0$ = 0.1 mM; $[HA]_0$ = 1 mM; 0.1 mM phosphate buffer at pH_0 = 7.0; reaction time = 1 h).*

The reduction of polysaccharide was generally higher than that of protein by all of the treatments (Nspd, Cu(II), Cu(II)+Nspd, Cu(II)/HA, Cu(II)/HA+Nspd) (Fig. 3. 3). Cu(II) exhibits the least decrease on the bioarea of protein and polysaccharides, but that of Cu(II) incorporated with Nspd was increased compared with that of Cu(II). Cu(II)/HA can produce reactive oxidant that actively reacts with EPS, thus it can reduce protein and polysaccharides. However, the produced oxidant of the Cu(II)/HA/Nspd system would be reduced by the complexation with Nspd. This presumably contributed to the enhanced efficacy of biofilm inactivation. Nspd actuated biofilm inactivation by the Cu(II)/HA system besides the reduction of EPS. This assumption could be supported by the results that the inactivation efficacy of Nspd grown biofilm, which grew up in nutrients containing Nspd during a continuous-flow culture, was reduced compared with that of the Nspd and Cu(II)/HA co-added system (Fig. 3. 5). Nspd grown biofilm shows higher inactivation efficacy than the normal grown one by the Cu(II)/HA system, while Cu(II) treatment exhibits similar efficacy for both normal and Nspd grown biofilm.

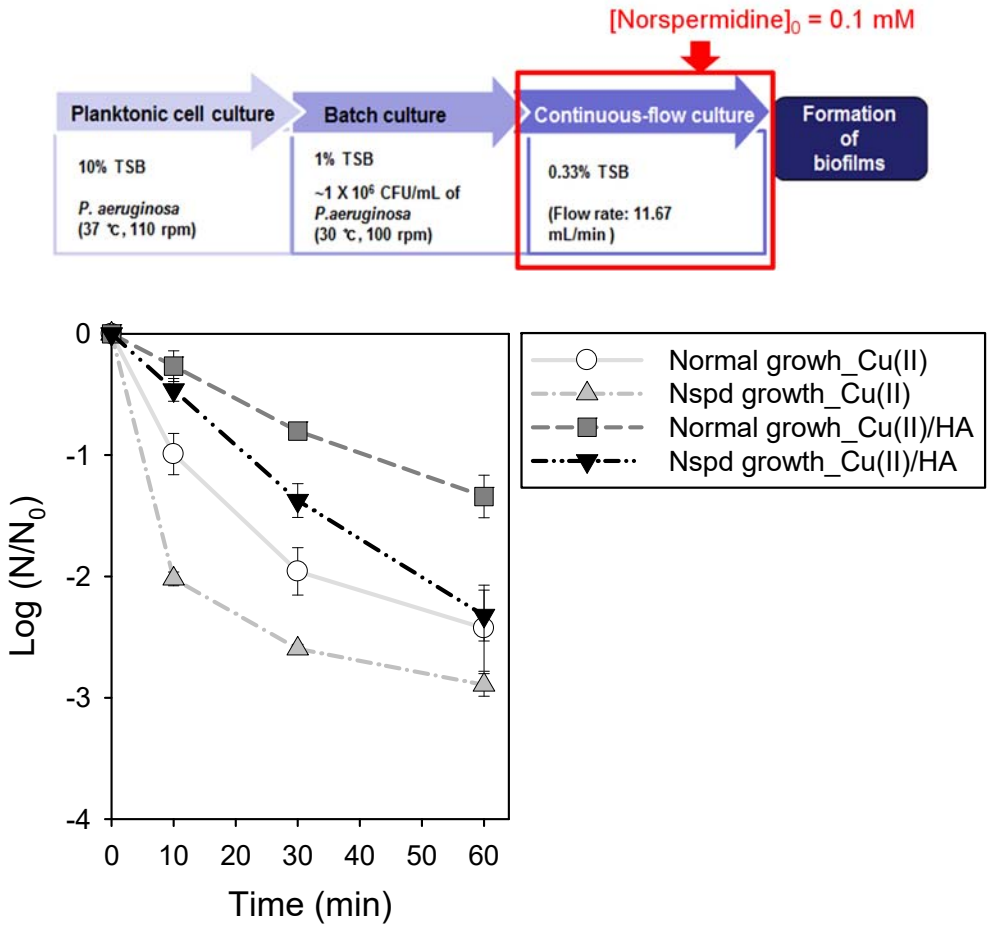

Figure 3. 5 *Effects of norspermidine treatment during the continuous-flow culture on biofilm inactivation by the Cu(II) and Cu(II)/HA system (Initial cell concentration: ~1 × 10^9, ~1 × 10^8 CFU/cm^2; $[Norspermidine]_0$ = 0.02, 0.1, 0.5 mM; $[Cu(II)]_0$ = 0.1 mM; $[HA]_0$ = 1 mM; 0.1 mM phosphate buffer at pH_0 = 7.0; reaction time = 1 h).*

Detached culturable cells and total cells were examined on the coupon, so that it was recognizable to count the number of detached total cells as well as the culturable proportion in detached total cells. From Figs. 3. 6a and b, it appears that the total cells that remain on the coupon were dissociated only less than 0.5 log (50%) degree for all of the treated conditions except Cu(II) treatment, despite that of culturable cells varying from 0 to 4 log. This indicates that the concentration of the injected Nspd was not enough to drive the detachment of biofilm. It is noteworthy that the total cell by the only Nspd treatment was reduced to the comparable level to that of a combination of Nspd and copper. Hence, reduction of culturable cell by the Nspd treatment could result in detachment of the cell rather than inactivation.

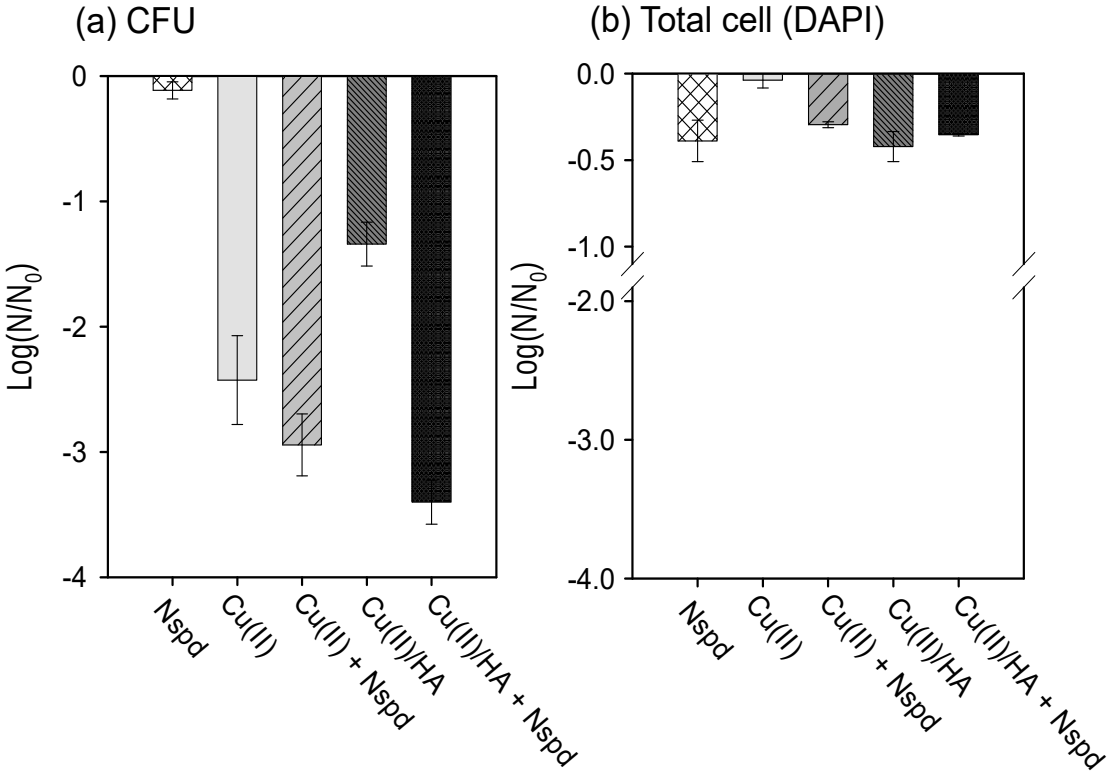

Figure 3. 6 *Culturable (a) and total cells (b) on the coupon and in bulk by the treatment of Nspd with Cu(II) and Cu(II)/HA (Initial cell concentration: ~1 × 10^9 CFU/cm^2; $[Nspd]_0$ = 0.1 mM; $[Cu(II)]_0$ = 0.1 mM; $[HA]_0$ = 1 mM; 0.1 mM phosphate buffer at pH_0 = 7.0; reaction time = 1 h; 100 rpm).*

Collectively, improved inactivation of biofilm by Cu(II)/HA in combination with Nspd was attributed to two major aspects. i) The addition of Nspd leads to hastened penetration of Cu(I), which produces the Cu(II)/HA system. Nspd could induce the collapse of EPS structure in biofilm to make Cu(I) accessible, which was blocked by biofilm structural barrier (reduction of protein and polysaccharides in Fig. 3. 3). ii) It is plausible that Nspd can act as a stabilizer of Cu(I), and result in lower reactivity to the biofilm component, which means the produced oxidant will be much less. Reduced reactivity by the complexation with Nspd could accelerate the diffusion rate of the disinfectant. Furthermore, Cu(I) is highly unstable, and easy to oxidize into Cu(II) under oxic condition.

3.2.3 Inactivation of biofilms on RO membranes in a cross-flow filtration unit

The cell population on RO membranes, the permeate flux, and the salt rejection were examined by Cu(II)/HA + Nspd treatment on the inactivation of biofilms grown for 24 h on RO membrane in a cross-flow filtration unit (Fig. 3. 7). During the first 24 h before the treatment, the biofilm formation on RO membranes remarkably declined the permeate flux, and slightly decreased salt rejection. At the point of 24 h biofouling occurrence, the Cu(II)/HA+Nspd treatment was conducted for another 4 h. By the treatment, *P. aeruginosa* cells were rapidly inactivated from approximately $10^{8.94}$ to $10^{6.02}$ CFU/cm^2, and this result shows much higher inactivation efficacy compared to the inactivation results in the absence of Nspd condition in a recent paper we published (e.g. Cu(II)/HA treatment shows only 50% cell inactivation under identical conditions). In addition, the permeate flux was recovered to 50-60% of the initial flux with delayed time. The results indicate that Cu(II)/HA/Nspd treatment is also applicable to in situ cleaning for membrane biofouling. According to previous study related to biofouling on membrane, recovery of water flux increased with increase of D-tyrosine (one of the signal molecules) concentration (Xu and Liu, 2011). This implies that higher concentration of Nspd than the tested condition could induce more biofilm dispersion from the membrane surface, and prompt further enhancement of flux recovery.

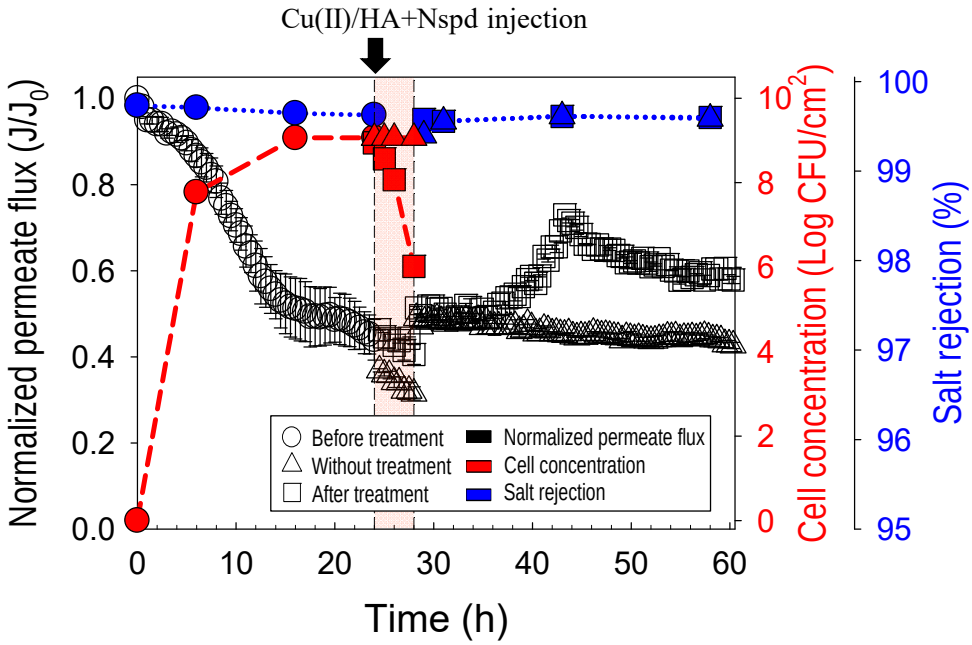

Figure 3. 7 *Variations in normalized permeate flux, salt rejection, and bacterial cell concentration in biofilms in a cross-flow filtration unit: biofilms were grown for 24 h, and subsequently disinfectants were injected (Initial permeate flux: 55 L m^{-2} h^{-1}; Pressure: 15.5 bar; [Cu(II)]$_0$ = 0.1 mM; [Nspd]$_0$ = 0.1 mM; [HA]$_0$ = 1 mM).*

3.3 Summary

This study investigated the biofilm control on RO membrane by the copper ion in combination with norspermidine. The major findings of this study are summarized as follows.

- Combination with Nspd in the Cu(II) and Cu(II)/HA system inhibited inactivation of the planktonic cell. In contrast, the addition of Nspd significantly enhanced the inactivation of *P. aeruginosa* cells in biofilms, especially in the Cu(II)/HA system.

- The Nspd-added copper based system appeared to reduce protein and polysaccharides with marginally minimization of the concentration of total cells. The results indicate that the concentration of applied Nspd was insufficient to detach biofilm cells, but helped to disrupt EPS (protein and polysaccharides), to enhance the penetration of copper-based disinfectants (scheme for the suggested inactivation mechanism: Fig. 3. 8).

- From a practical point of view, the Cu(II)/HA/Nspd treatment is effectively utilized to inactivate cells in biofilms on RO membranes in the cross-flow filtration unit. In addition, this shows the potential as membrane cleaning agents, since the treatment substantially recovered permeate flux.

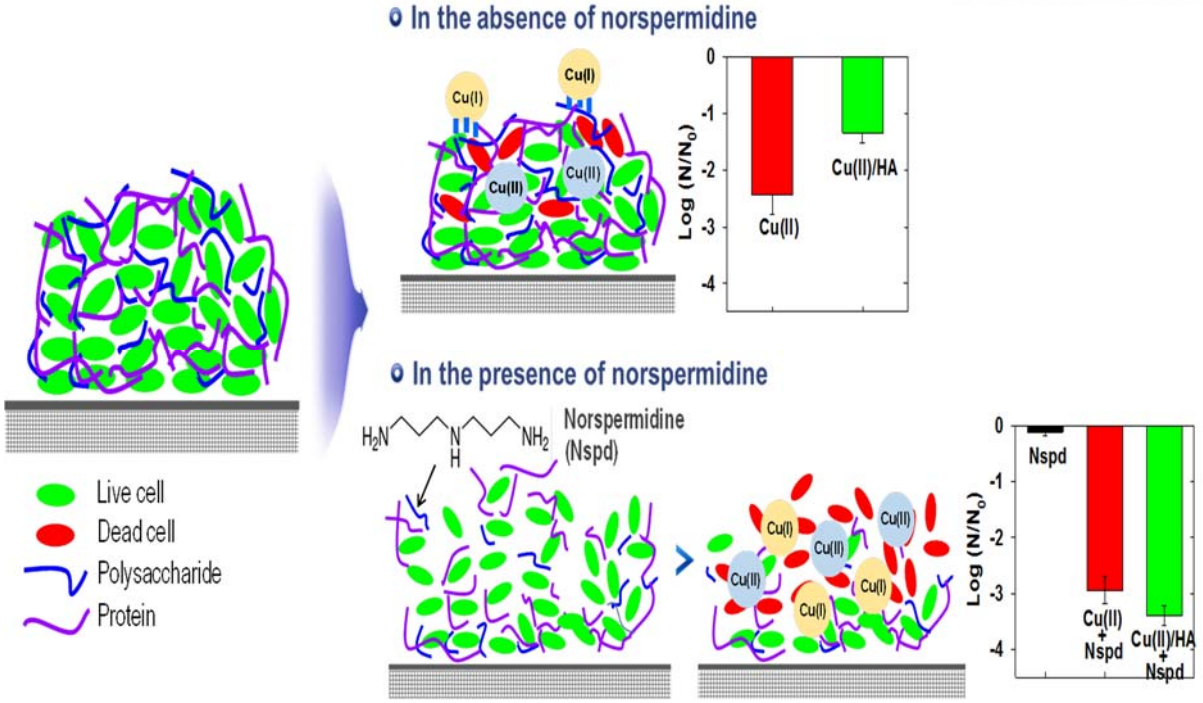

Figure 3. 8 *Scheme for suggested inactivation mechanism by the copper ion in combination with norspermidine.*

IV. INACTIVATION OF PLANKTONIC AND BIOFILM CELLS BY CU(II)-ACTIVATED PERSULFATE IN THE PRESENCE OF CHLORIDE ION

4.1 Materials and methods

4.1.1 Reagents

Microorganism culture reagents, including tryptic soy broth (TSB) and tryptic soy agar (TSA), were obtained from Becton-Dickinson Co. (USA). Copper sulfate ($CuSO_4$), potassium peroxymonosulfate (PMS, $KHSO_5$ as Oxone, $2KHSO_5 \cdot KHSO_4 \cdot K_2SO_4$, DuPont Co. USA), ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA-Na_2), sodium chloride (NaCl), sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$), sodium hypochlorite solution (NaOCl, 10-15% active chlorine), methanol, and phenol was purchased from Sigma-Aldrich Co. (USA). All chemicals were of reagent grade and used without additional purification. All glassware was deionized water and sterilized by autoclave at 121 °C for 15 min prior to use.

4.1.2 Culture and analysis of *P. aeruginosa*, and inactivation experiments of planktonic cells

Culture and analysis of *P. aeruginosa* was carried out in the same manner described in a previous chapter. All experiments were performed in a batch system at room temperature (22 ± 2°C) using a 60-mL Pyrex reactor open to the atmosphere. One-milliliter of solution was sampled from the reactor at the predetermined time, and immediately quenched with EDTA and sodium thiosulfate.

4.1.3 Biofilm in CDC reactors and a cross-flow filtration unit

Biofilms of *P. aeruginosa* were grown in center for disease control (CDC) biofilm reactors (CBR 90, Biosurface Technologies Co., USA). Detail of reactor feature and conditions for biofilm growth was described in previous chapter, and performed in the same manner.

Biofilm experiments using a cross-flow filtration unit was carried out under the same condition described in previous chapter. The biofilm inactivation experiment was initiated by injecting reagents (Cu(II), PMS) into the feed water tank.

4.1.4 Analytical methods

Stock solutions of chlorine (100 ppm) were prepared by diluting a commercial solution of sodium hypochlorite. Stock solutions of each oxidant were standardized spectrophotometrically based on their molar absorption coefficient: $\varepsilon = 350\ M^{-1}\ cm^{-1}$ at 292 nm for OCl^- (Johnson and Margerum, 1991). Free chlorine was measured by the DPD colorimetric method (4500-Cl G) (APHA et al., 1995).

Phenol and benzoic acid were analyzed using a rapid separation liquid chromatography (RSLC) (UltiMate 3000; Thermo Fisher Scientific Inc., USA) with a UV absorbance detection at 277 and 227 nm, respectively. Separation was performed on an Acclaim™ RSLC 120 C18 column (50 mm × 2.1 mm, 2.2 μm; Thermo Fisher Scientific Inc., USA). A mixture of 0.1% (w/w) phosphoric acid/acetonitrile (50%/50% (v/v) for phenol and benzoic acid) was used as the mobile phase at a 0.5 mL min^{-1} flow rate.

4.2 Results and Discussion

4.2.1 Inactivation of planktonic cells by PMS-activated disinfection system

The inactivation of planktonic *P. aeruginosa* cells was examined by different PMS-activated disinfection systems (i.e., PMS, Cu(II), Cu(II)/PMS, PMS/Cl^-, and Cu(II)/PMS/Cl^- systems) (Fig. 4. 1). PMS, Cu(II), and PMS/Cl^- system exhibited less than 0.5 log inactivation. In contrast, the inactivation efficacy was greatly enhanced by applying Cu(II) to activate PMS in the Cu(II)/PMS and Cu(II)/PMS/Cl^- systems. It is interesting to note that the Cu(II)/PMS/Cl^- system (3.1 log in 10 min) shows higher inactivation efficacy than the Cu(II)/PMS system (3.4 log in 20 min), although the PMS/Cl^- system shows negligible inactivation.

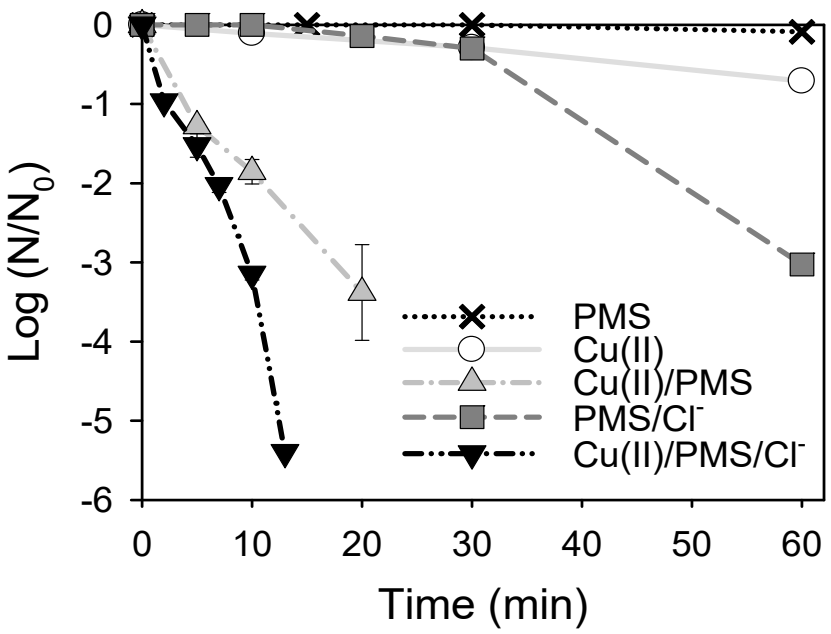

Figure 4. 1 *Inactivation of planktonic P. aeruginosa cells by different PMS-activated disinfection systems (Initial cell concentration: ~1 × 10^7 CFU/mL; $[Cu(II)]_0$ = 5 μM; $[PMS]_0$ = 0.2 mM; $[NaCl]_0$ = 10 mM; 1 mM phosphate buffer at pH_0 = 7.0).*

Reactive oxidants such as $SO_4^{\bullet-}$ and $^{\bullet}OH$ can be produced via activation of PMS by cupric ion (reaction 1-3). Phenol and benzoic acid was selected as chemical probes to identify the primary radical formed by the Cu(II)-activated PMS system. $SO_4^{\bullet-}$ can decompose phenol 7.3 times faster than benzoic acid, $^{\bullet}OH$ reacts with both compounds at high rate (Table 4.1) (Liang and Su, 2009). Phenol was completely decomposed by the Cu(II)/PMS system for 6 h reaction under the ~1 × 10^9 CFU/mL condition (Fig. 4. 2), but benzoic acid was not degraded by different PMS-activated disinfection systems (i.e., PMS, Cu(II)/PMS, PMS/Cl^-, and Cu(II)/PMS/Cl^- systems) under identical conditions (Fig. 4. 3). The results indicate that $SO_4^{\bullet-}$ is predominantly responsible for the inactivation, rather than $^{\bullet}OH$. In addition, the target affinity on bacteria cells would also accelerate phenol degradation.

$$HSO_5^- + Cu(I) \rightarrow Cu(II) + SO_4^{\bullet-} + OH^- \quad (1)$$

$$HSO_5^- + Cu(I) \rightarrow Cu(II) + SO_4^{2-} + {}^{\bullet}OH \quad (2)$$

$$HSO_5^- + Cu(II) \rightarrow Cu(I) + SO_5^{\bullet-} + H^+ \quad (3)$$

Table 4. 1 *Second-order rate constants for the reaction of $SO_4^{\bullet-}$ and $^{\bullet}OH$ with target compound*

Compound	$SO_4^{\bullet-}$	$^{\bullet}OH$
Phenol	8.8×10^9 M^{-1} s^{-1}	6.6×10^9 M^{-1} s^{-1}
Benzoic acid	1.2×10^9 M^{-1} s^{-1}	4.2×10^9 M^{-1} s^{-1}

Fig. 4. 2 shows that degradation of phenol by the Cu(II)/PMS system increases with increasing population of *P. aeruginosa* cells. It is presumed that sulfate radical reaction most likely occurs nearby the bacteria rather than in bulk. Previous literature demonstrated that Cu(II) can reduce into Cu(I) in *E. coli* cells using intracellularly generated superoxide radical (Park et al., 2012). In general, reduction of metal catalysis is much slower than the oxidation reaction. Accordingly, the sulfate radial production reaction is likely induced around bacteria where the Cu(I) was interred, and the produced bacteria-bounded sulfate radical would directly damage *P. aeruginosa* cells.

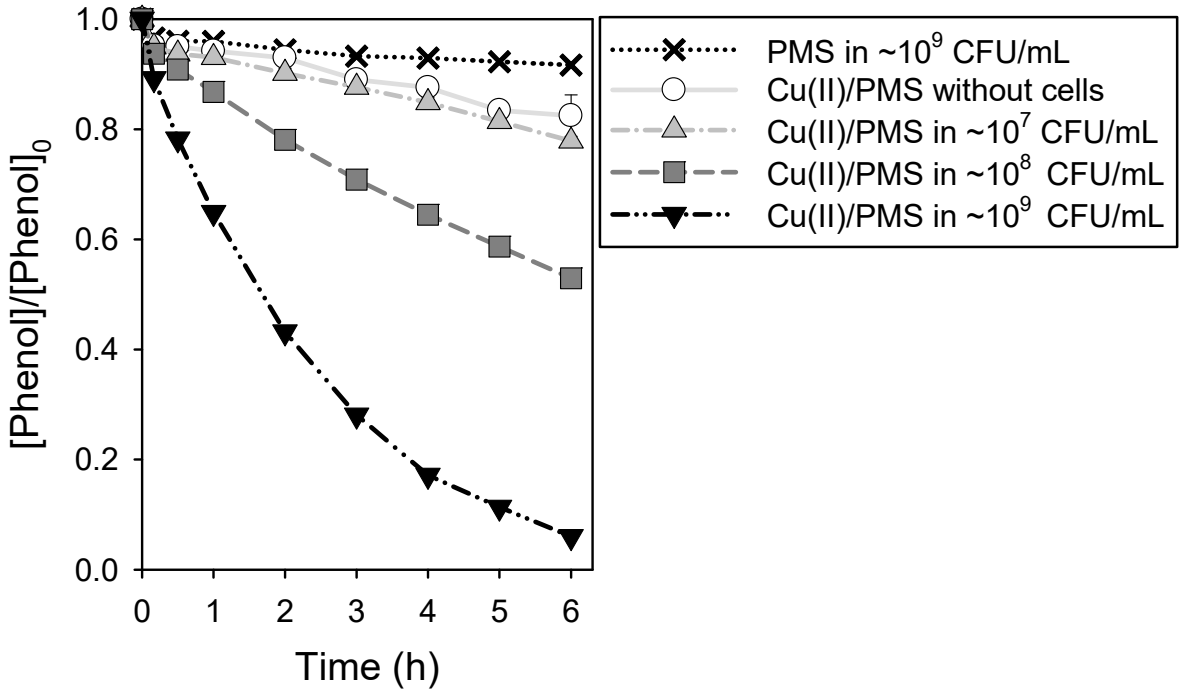

Figure 4. 2 *Effect of planktonic P. aeruginosa cells on degradation of phenol by the Cu(II)/PMS system (Initial cell concentration: ~1 × 10^7, ~1 × 10^8, ~1 × 10^9 CFU/mL; $[phenol]_0$ = 10 μM; $[Cu(II)]_0$ = 0.1 mM; $[PMS]_0$ = 1.0 mM; $[NaCl]_0$ = 50 mM; 10 mM phosphate buffer at pH_0 = 7.0).*

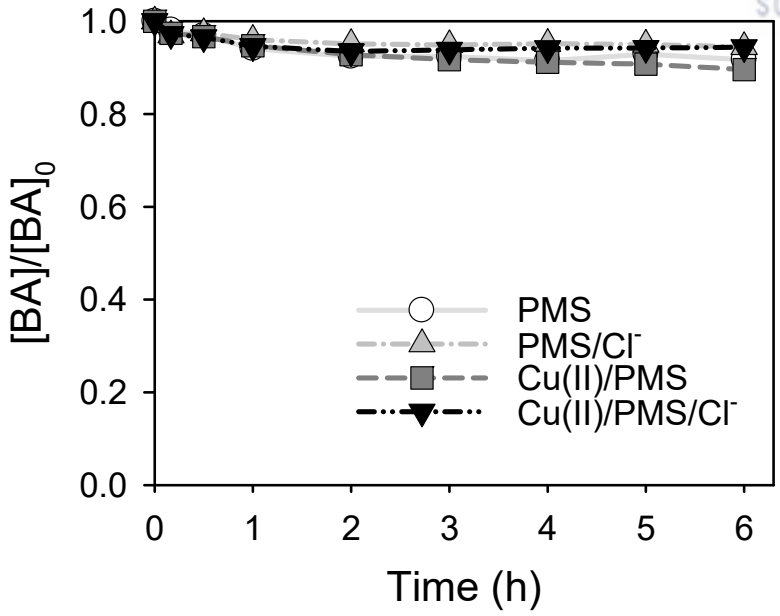

Figure 4. 3 *Degradation of benzoic acid by different PMS-activated disinfection systems (Initial cell concentration: ~1 × 10^9 CFU/mL; [benzoic acid]$_0$ = 10 μM; [Cu(II)]$_0$ = 0.1 mM; [PMS]$_0$ = 1.0 mM; [NaCl]$_0$ = 50 mM; 10 mM phosphate buffer at pH$_0$ = 7.0).*

A series of experiments including inactivation efficacy, and production of chlorine were conducted in order to elucidate the effect of chloride ion in PMS-activated disinfection systems. Firstly, the bactericidal activities of Cu(II)/Cl^-, PMS/Cl^-, and Cu(II)/PMS/Cl^- systems were observed as a function of chloride ion dose (Fig. 4. 4). The inactivation efficacy by the PMS/Cl^- system was gradually enhanced with increasing concentration of chloride ion, while the Cu(II)/Cl^- system did not show any difference. It has been known that the PMS/Cl^- system can produce active chlorine through the reaction 4-5, and the inactivation phenomenon using the PMS/Cl^- system has been reported (Anipsitakis et al., 2008; Delcomyn et al., 2006).

$$HSO_5^- + Cl^- \rightarrow H^+ + OCl^- + SO_4^{2-} \quad k = 1.35 \times 10^{-3}\ (M^{-1}S^{-1}) \qquad (4)$$

$$HSO_5^- + 2Cl^- + H^+ \rightarrow SO_4^{2-} + Cl_2 + H_2O \qquad (5)$$

With the increase of chloride ion concentration, the inactivation efficacy of the Cu(II)/PMS/Cl^- system was also rapidly enhanced. The only difference between Figs. 4. 4b and c is the activation of copper. This result indicates that Cu(II) is important to activate PMS as well as HOCl. In fact, sulfate radical involved reactions for the production of chlorine (reaction 6-9) are well established in previous literature by employing cobalt ion to activate PMS (Anipsitakis et al., 2006; Yuan et al., 2011).

$$SO_4^{\bullet -} + Cl^- \leftrightarrow SO_4^{2-} + Cl^\bullet \quad k = 4.7 \times 10^8\ (M^{-1}S^{-1}) \qquad (6)$$

$$Cl^\bullet + Cl^- \rightarrow Cl_2^{\bullet -} \quad k = 8 \times 10^9\ (M^{-1}S^{-1}) \qquad (7)$$

$$Cl_2^{\bullet -} + Cl_2^{\bullet -} \rightarrow Cl_2 + 2Cl^- \quad k = 9 \times 10^8\ (M^{-1}S^{-1}) \qquad (8)$$

$$Cl^\bullet + Cl^\bullet \rightarrow Cl_2 \quad k = 8.8 \times 10^7\ (M^{-1}S^{-1}) \qquad (9)$$

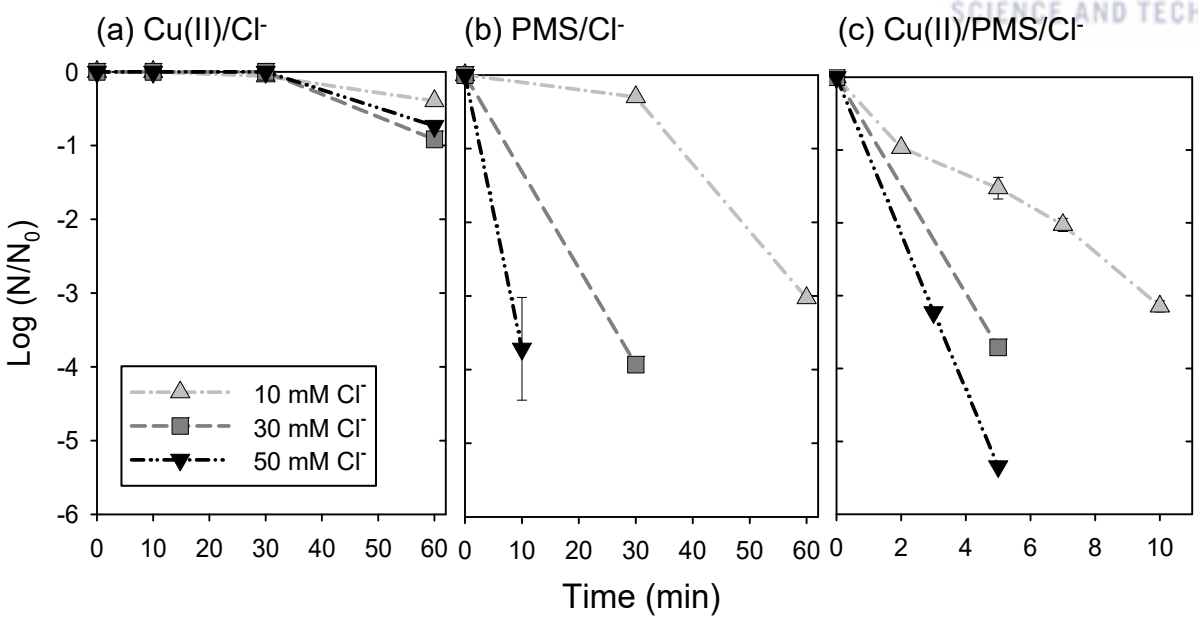

Figure 4. 4 *Effect of chloride ion on inactivation of planktonic P. aeruginosa cells by different PMS-activated disinfection system (Initial cell concentration: ~1 × 10^7 CFU/mL; $[Cu(II)]_0$ = 5 μM; $[PMS]_0$ = 0.2 mM; $[NaCl]_0$ = 10, 30, 50 mM; 1 mM phosphate buffer at pH_0 = 7.0).*

The chlorine generated by the PMS/Cl^- and Cu(II)/PMS/Cl^- system was measured to investigate the effect of copper ion for the in situ production of active chlorine (Fig. 4. 5). Production of chlorine was hardly observed in the absence of chloride ion (i.e., PMS, Cu(II)/PMS). In contrast, active chlorine was observed in the presence of chloride ion (i.e., PMS/Cl^-, Cu(II)/PMS/Cl^-), and appeared to be a more or less similar trend in the absence and presence of copper in Fig. 4. 5. The production yield of chlorine was 0.03% regardless of the concentration of chloride ion. There are two ways (non-radical and radical pathway) for the in situ generation of active chlorine. PMS can oxidize chloride ion to active chlorine species in non-radical pathways (reaction 4-5). There are also sulfate radical-involved pathways to produce active chlorine (reaction 6-9). This result indicates that sulfate radical related reaction for the production of active chlorine is a relatively minor pathway compared to the non-radical pathway.

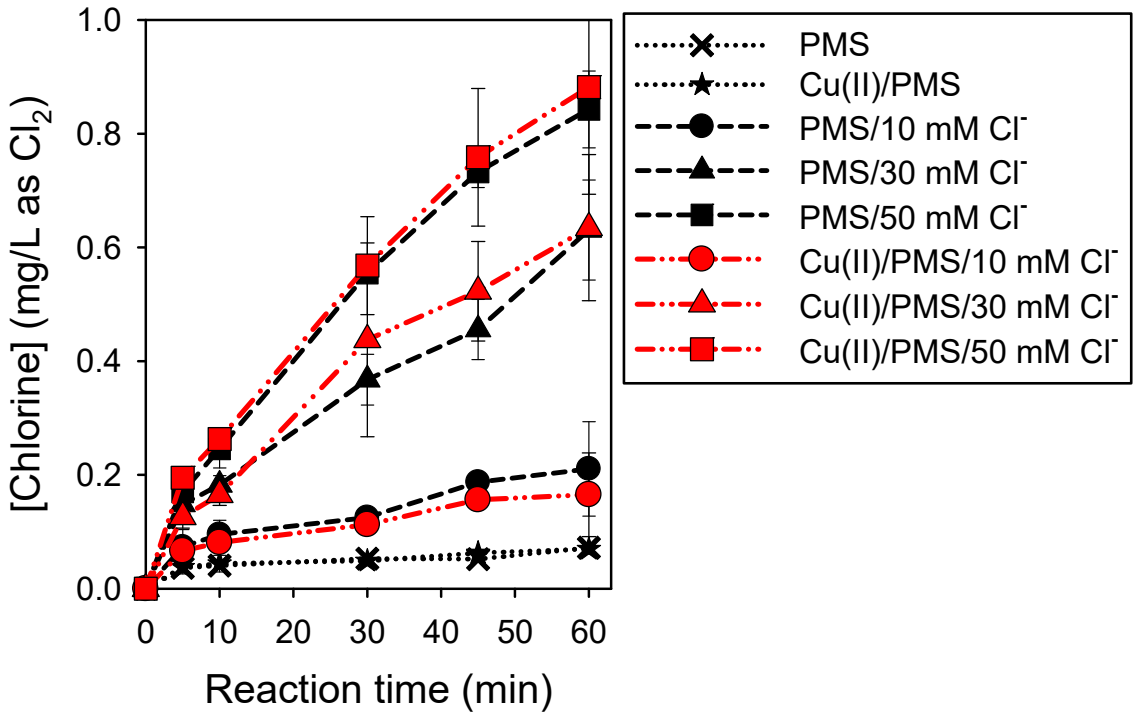

Figure 4. 5 *Production of chlorine by different PMS-activated disinfection system ($[Cu(II)]_0$ = 5 μM; $[PMS]_0$ = 0.2 mM; $[NaCl]_0$ = 10, 30, 50 mM; 1 mM phosphate buffer at pH_0 = 7.0).*

The inactivation efficacy by chlorine was examined to clarify the role of chlorine in the Cu(II)/PMS/Cl^- system (Fig. 4. 6a). This only achieved less than 1 log inactivation by the addition of 0.1 ppm chlorine, but *P. aeruginosa* cells were inactivated to 4-5 log with chlorine higher than 0.1 ppm (i.e., 0.2, 0.3 ppm). Inactivation by in situ produced chlorine was not supposed to occur under the condition with 10 mM NaCl for 30 min (less than 0.1 ppm of chlorine produced, as shown in Fig. 4. 5), because 0.1 ppm is not enough concentration to derive the inactivation. This observation implies that additional reactive species capable of causing inactivation might be produced by the Cu(II)/HOCl system through reaction 10-12. Indeed, the inactivation efficacy of chlorine was greatly enhanced by the combination with Cu(II) as shown in Fig. 4. 6b. Overall, the enhanced inactivation efficacy in the Cu(II)/PMS/Cl^- system was attributed to the synergistic effect of two factors, i) production of sulfate radical by the Cu(II)/PMS system, and ii) formation of reactive species by the Cu(II)/in situ produced HOCl system.

$$HOCl + Cu(I) \rightarrow Cu(II) + {}^{\bullet}OH + Cl^- \quad (10)$$

$$HOCl + Cu(I) \rightarrow Cu(II) + OH^- + {}^{\bullet}Cl \quad (11)$$

$$HOCl + Cu(I) \rightarrow Cu(III) + OH^- + Cl^- \quad (12)$$

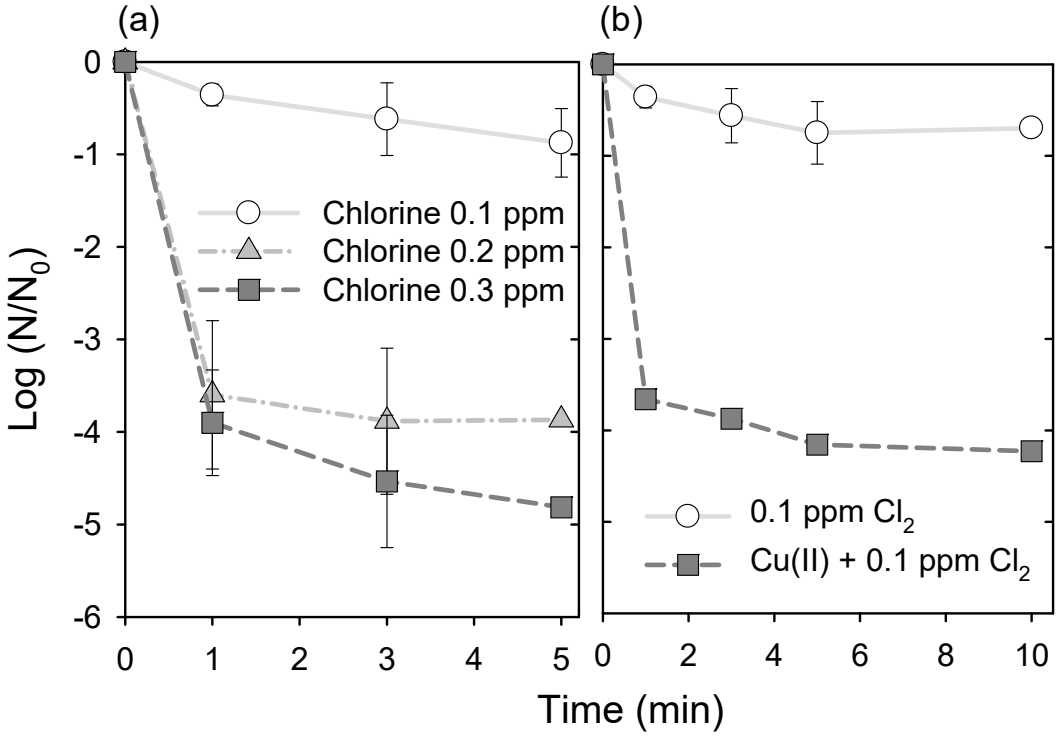

Figure 4. 6 *Inactivation of planktonic P. aeruginosa cells by chlorine (a) and Cu(II)/chlorine (b) (Initial cell concentration: ~1 × 10^7 CFU/mL; [Chlorine]$_0$ = 0.1, 0.2, 0.3 ppm; 1 mM phosphate buffer at pH$_0$ = 7.0).*

4.2.2 Inactivation of biofilms on RO membranes by PMS-activated disinfection system

The inactivation efficacy of biofilm formed in CDC reactors was examined by different PMS-activated disinfection systems (i.e., PMS, Cu(II), Cu(II)/PMS, PMS/Cl^-, and Cu(II)/PMS/Cl^- systems) (Fig. 4. 7). Typically, inactivation of biofilm by the Cu(II)/PMS system was found to be more resistance than that in status of planktonic cells. Inactivation efficacy by the different PMS activated disinfection systems appeared in the order of PMS (1.2 log) < PMS/Cl^- (1.9 log) < Cu(II) (2.4 log) < Cu(II)/PMS/Cl^- systems (4.6 log) ≈ Cu(II)/PMS (4.7 log) during 1 h of reaction time (Fig. 4. 7).

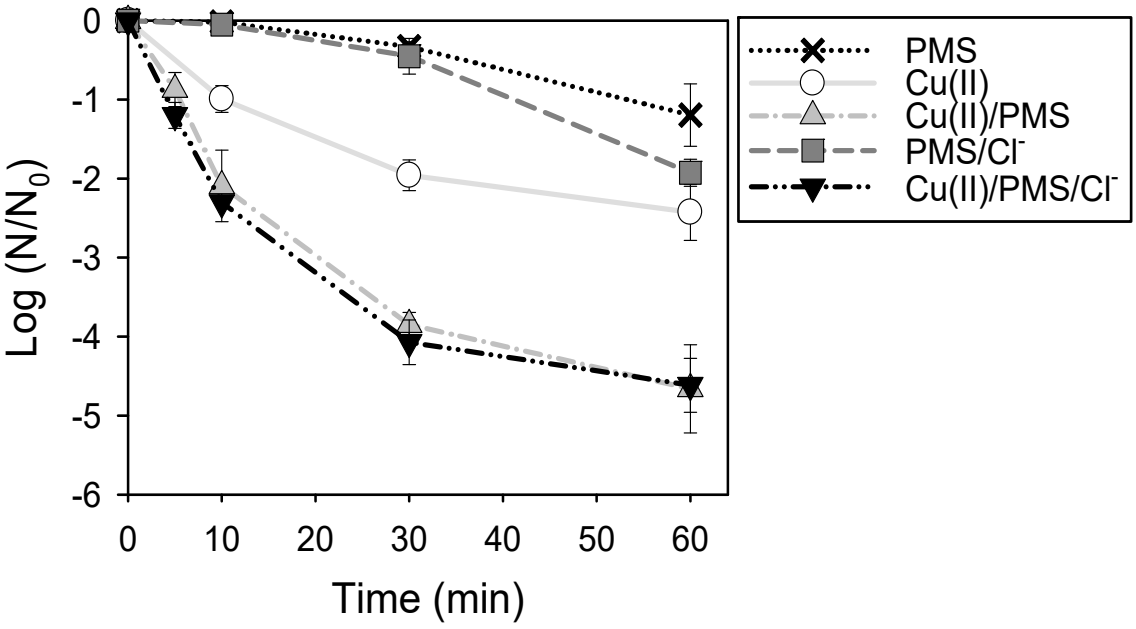

Figure 4. 7 *Inactivation of P. aeruginosa cells in biofilms by different PMS-activated disinfection system (Initial cell concentration: ~1 × 10^9 CFU/cm^2; $[Cu(II)]_0$ = 0.1 mM; $[PMS]_0$ = 1.0 mM; $[NaCl]_0$ = 10 mM; 1 mM phosphate buffer at pH_0 = 7.0).*

Enhanced inactivation efficacy was observed by the combination of Cu(II) and PMS in biofilm condition, and this phenomenon is constant in planktonic cells. In contrast, the Cu(II)/PMS and Cu(II)/PMS/Cl^- systems did not exhibit any difference of inactivation efficacy of biofilms, while the inactivation results of Cu(II)/PMS/Cl^- in planktonic cells exhibited significant enhancement compared to the Cu(II)/PMS. These results were caused by suppression of the synergistic effect of Cu(II)/chlorine due to the consumption of chlorine by EPS components. Biofilm was known to be 10,000 times more resistant to chlorine than that for planktonic cells for *P.aeruginosa* PAO1 (Kim et al., 2008). EPS components in the surface of biofilm rapidly reacted with chlorine, and diffusion of the disinfectant into the biofilm was limited (Stewart et al., 2001). Therefore, chlorine production in the Cu(II)/PMS/Cl^- system is only beneficial to the inactivation of planktonic cells, but it was not effective in inactivating biofilms.

4.2.3 Inactivation of biofilms on RO membranes in a cross-flow filtration unit

Inactivation of biofilm, which had grown on RO membrane in a cross-flow filtration unit, was performed by the Cu(II)/PMS system, and its efficacy was evaluated by analyzing the cell population, the permeate flux, and salt rejection (Figs. 4. 8 and 4. 9). As the cell concentration on RO membranes increases, the permeate flux gradually declined during 24 h of biofilm growth. The Cu(II)/PMS disinfection system was then applied for 4 h treatment. The Cu(II)/PMS treatment exhibited inactivation from approximately 10^9 to $10^{6.1}$ CFU/cm^2 at 2 h, and $10^{4.4}$ CFU/cm^2 at 4 h treatment (Fig. 4. 8), although the experiment was conducted at considerably low concentration (0.2 mM) of PMS. Salt rejection during the treatment slightly decreased from 99.7% to 99.5%.

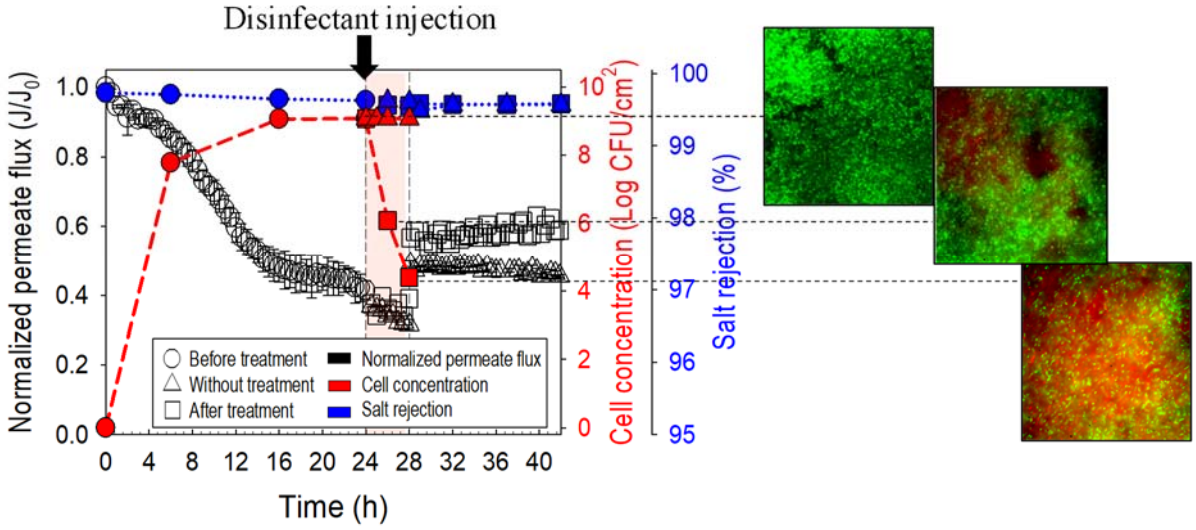

Figure 4. 8 *Variations in normalized permeate flux, salt rejection, and bacterial cell concentration in biofilms in a cross-flow filtration unit: biofilms were grown for 24 h, and subsequently disinfectants were injected (Initial permeate flux: 55 L m^{-2} h^{-1}; Pressure: 15.5 bar; [Cu(II)]$_0$ = 0.1 mM; [PMS]$_0$ = 0.2 mM; [NaCl]$_0$ = 10 mM).*

The Cu(II)/1.0 mM PMS treatment exhibited a significant degree of cell inactivation (9 log inactivation in 4 h; approximately 10^9 to $10^{3.1}$ CFU/cm^2 at 2 h, $10^{1.8}$ CFU/cm^2 at 4 h) (Fig. 4. 9). CLSM images show that cells in biofilms are mostly dead (indicated by cell membrane damage) after two hours of exposure to the Cu(II)/PMS system, but the biofilm structure was hardly disrupted as shown in Fig. 4. 10 in spite of the fact that 6 log inactivation was achieved. After 4 h of treatment, most of the dead cells are destructed (Fig. 4. 10). Recovery of permeate flux was achieved to approximately 70% by Cu(II)/1.0 mM PMS system. In general, inactivation efficacy and recovery of permeate flux improved in higher concentration of PMS injected condition (1.0 mM) compared to the results of 0.2 mM PMS condition (Fig. 4. 8).

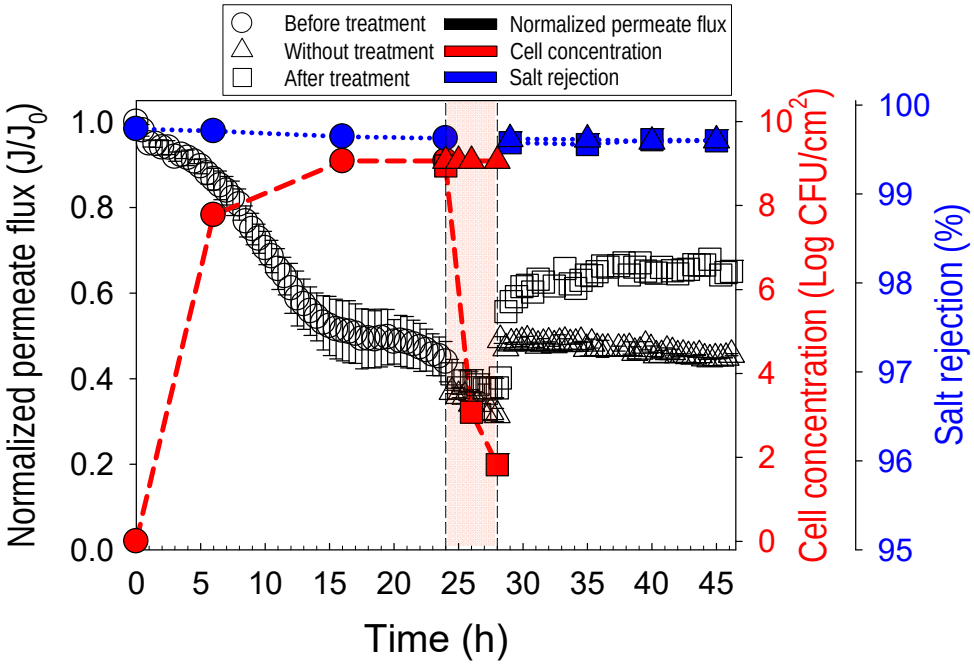

Figure 4. 9 *Variations in normalized permeate flux, salt rejection, and bacterial cell concentration in biofilms in a cross-flow filtration unit: biofilms were grown for 24 h, and subsequently disinfectants were injected (Initial permeate flux: 55 L m^{-2} h^{-1}; Pressure: 15.5 bar; [Cu(II)]$_0$ = 0.1 mM; [PMS]$_0$ = 1.0 mM; [NaCl]$_0$ = 10 mM).*

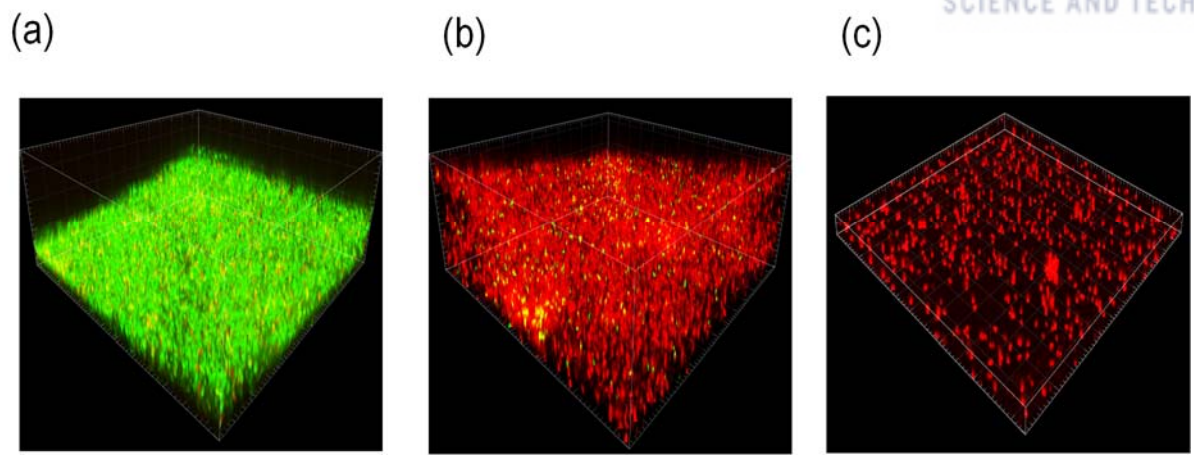

Figure 4. 10 *CLSM images of Cu(II)/PMS-treated P. aeruginosa biofilms on RO membranes in a cross-flow filtration unit: (a) before treatment (24 h biofilm growth), (b) after 2 h treatment and (c) after 4 h treatment (scale: 126.7 μm × 126.7 μm × 15-25 μm, live and dead cells are green and red, respectively).*

4.3 Summary

Inactivation of planktonic and biofilm cells by Cu(II)-activated persulfate was examined in the presence of chloride ion, and the inactivation mechanism was elucidated. The major findings of this study are summarized as follows.

- Inactivation efficacy was greatly enhanced by applying Cu(II) to activate PMS in the Cu(II)/PMS and Cu(II)/PMS/Cl^- systems for both planktonic and biofilm cells. It was found that the $SO_4^{\cdot -}$ is mainly produced by the Cu(II)-activated persulfate system. In addition, the production of sulfate radial is likely induced around bacteria where the Cu(I) was interred, and the produced bacteria-bound sulfate radical would directly damage *P. aeruginosa* cells.

- The Cu(II)/PMS/Cl^- system exhibits significantly higher inactivation efficacy than the Cu(II)/PMS system in planktonic cells. Enhanced inactivation efficacy in the Cu(II)/PMS/Cl^- system was attributed to the synergistic effect of the production of reactive species by the Cu(II)/PMS system and the Cu(II)/in situ produced HOCl system.

- In contrast, the Cu(II)/PMS and Cu(II)/PMS/Cl^- systems did not show any difference in the inactivation efficacy of biofilms by suppression of the synergistic effect of the Cu(II)/HOCl system due to the consumption of chlorine by EPS components.

- The Cu(II)/ PMS treatment in a cross-flow filtration unit exhibited a significant degree of cell inactivation (Fig. 4. 9). Recovery of permeate flux was achieved to approximately 70% by the Cu(II)/1.0 mM PMS system, verifying its applicability as a membrane cleaning reagent.

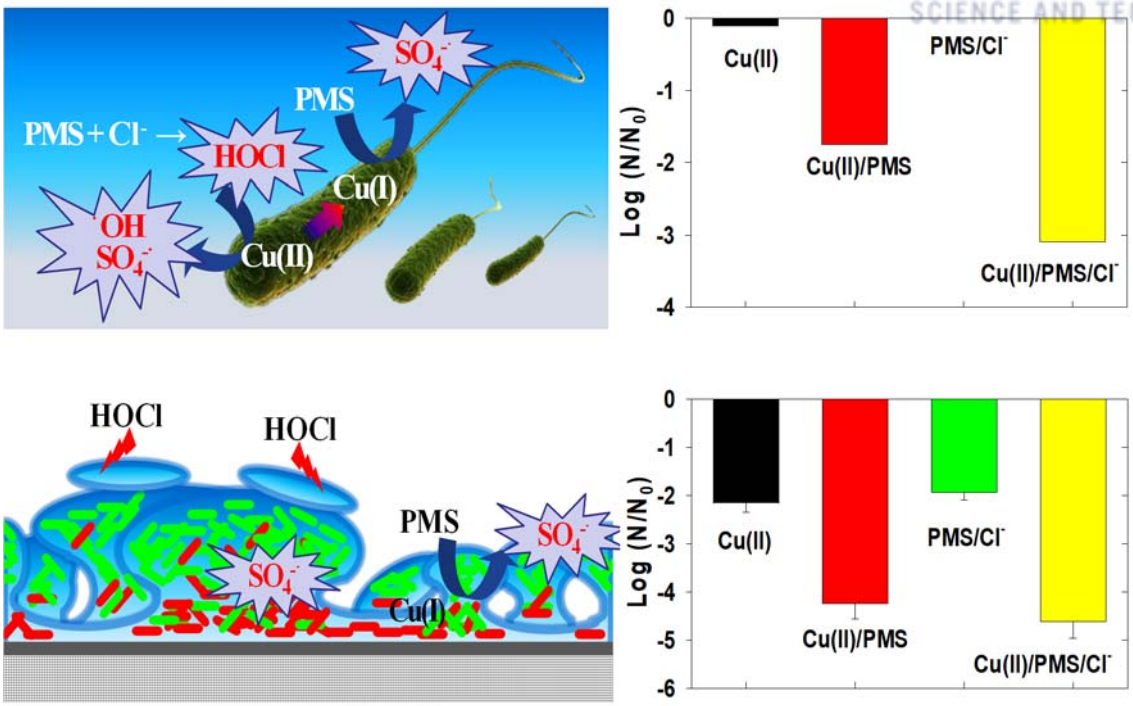

Figure 4. 11 *Scheme for suggested inactivation mechanism by Cu(II)-activated persulfate in the presence of chloride ion.*

V. CONCLUSIONS

This study evaluated the inactivation efficacy of cooper-based hybrid disinfection systems on planktonic and biofilm cells, and assessed the possibility of application as a disinfection reagent for pretreatment and membrane cleaning (clean-in-place).

In the first part of this dissertation, the biocidal activity of copper-based disinfection system was enhanced by the combination of cupric ion with HA and H_2O_2 compared to the utilization of one auxiliary reagent (HA or H_2O_2). The Cu(II)/HA/H_2O_2 system was successfully applied for pretreatment as well as membrane cleaning for biofouling control, while the Cu(II)/H_2O_2 and Cu(II)/HA systems appeared to be invalid.

In the second part of this dissertation, the biofilm inactivation by the copper ion was superior in combination with norspermidine. Nspd contributed to the disruption of EPS (especially polysaccharide), and allowed copper ion to penetrate into the inner part of biofilm. In addition, the Cu(II)/HA/Nspd system was capable of achieving 3 log (99.9%) biofilm inactivation under the pressurized condition as well as the recovery of permeate flux.

In the third part of this dissertation, the inactivation efficacy of Cu(II)-activated persulfate systems in planktonic cells was elevated in the presence of chloride ion via the production of chlorine. In contrast, the effect of chloride ion was found to be impaired for biofilm inactivation by the consumption of chlorine in the EPS component. The biofilms in pressurized condition were significantly inactivated by the Cu(II)/PMS system up to 9 log.

The capability of application to pretreatment and CIP treatment was evaluated based on the major finding (Table 1). A copper-based system in combination with HA and H_2O_2 is a reasonable choice for both pretreatment and CIP treatment. An Nspd-added system is applicable for CIP treatment for the inactivation of biofilms, whereas it was not suitable for pretreatment, considering that the inactivation efficacy was rather decreased in planktonic cells. An activated persulfate system can be applied in the presence of chloride ion as a pretreatment step, but because of the production of chlorine, it is inappropriate for CIP treatment.

Although the application results of this dissertation show that the salt rejection did not significantly drop after the injection of disinfectants, indicating that the RO membrane damage was not critical under the conditions employed, the stability of RO membranes (i.e., no oxidative damage by the treatment) should be investigated for the practical application in the RO membrane process. In addition, the long-term stability of RO membranes needs to be studied under conditions relevant to the particular uses of the disinfectants.

Table 5. 1 *Evaluation of disinfectants for pretreatment and CIP treatment in RO membrane process.*

Disinfectants		Pretreatment	CIP (clean-in-place)
Combination of cupric ion with hydroxylamine and hydrogen peroxide	Cu(II)	X	X
	$Cu(II)/H_2O_2$	X	X
	Cu(II)/HA	○	X
	$Cu(II)/HA/H_2O_2$	○	○
Copper ion in combination with norspermidine	Cu(II)/Nspd	X	○
	Cu(II)/HA/Nspd	X	○
Cu(II)-activated persulfate in the presence of chloride ion	Cu(II)/PMS	○	○
	PMS/Cl^-	○	X
	$Cu(II)/PMS/Cl^-$	○	X

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